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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12N 15/62, C07K 14/52, 19/00, 14/54,</b> <b>A61K 38/19, 38/20, 48/00, C12N 15/19,</b> <b>15/24, 15/13</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 96/23888</b> <b>(43) International Publication Date:</b> <b>8 August 1996 (08.08.96)</b>
<b>(21) International Application Number:</b> PCT/US96/00830 <b>(22) International Filing Date:</b> 1 February 1996 (01.02.96) <b>(30) Priority Data:</b> 08/383,035 3 February 1995 (03.02.95) US  <b>(60) Parent Application or Grant</b> <b>(63) Related by Continuation</b> US 08/383,035 (CIP) Filed on 3 February 1995 (03.02.95)  <b>(71) Applicant (for all designated States except US):</b> G.D. SEARLE & CO. [US/US]; Corporate Patent Dept., P.O. Box 5110, Chicago, IL 60680-5110 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> STATEN, Nicholas, R. [US/US]; 859 Queen Ann Place, St. Louis, MO 63122 (US). FAVARA, Jean, P. [US/US]; 142 Holly Garden Drive, Ballwin, MI 63021 (US). KAHN, Larry, E. [US/US]; 1030 Tempo Drive, St. Louis, MO 63146 (US). BAUM, Charles, M. [US/US]; 1712 Mason Knoll Road, St. Louis, MO 63131 (US). PEGG, Lyle, E. [US/US]; 1164 Oak Borough		<b>Drive, Chesterfield, MO 63017 (US). MCKEARN, John, P.</b> <b>[US/US]; 18612 Babler Meadows Drive, St. Louis, MO</b> <b>63038 (US).</b>  <b>(74) Agents:</b> BENNETT, Dennis, A. et al.; G.D. Searle & Co., Corporate Patent Dept., P.O. Box 5110, Chicago, IL 60680- 5110 (US).  <b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AZ, BY, KG, KZ, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> NOVEL c-MPL LIGANDS  <b>(57) Abstract</b>  The present invention relates to human c-mpl ligands (thrombopoieting) with activity on hematopoietic differentiation and expansion.		

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Novel c-MPL Ligands

This is a continuation-in-part of United States Application Serial No.08/383,035 filed February 04, 1995 which is incorporated herein by reference.

Field of the Invention

The present invention relates to human c-mpl ligands (thrombopoietin) with activity on hematopoietic differentiation and expansion.

Background of the Invention

Megakaryocyte (MK) maturation and platelet production has been long thought to be regulated by lineage specific humoral growth factors in a manner similar to cytokines that induce erythrocyte (erythropoietin) and granulocyte (G-CSF) expansion and maturation. Platelets are responsible for the prevention of bleeding in response to vascular injury. Therefore, platelet production is a vital component of hematopoietic regulation. Patients undergoing chemotherapy or bone marrow transplantation usually experience severely depressed platelet levels (thrombocytopenia) which may result in life threatening bleeding episodes. Several known growth factors and cytokines have been found to stimulate megakaryocytes and platelet production but most are pleiotropic both *in vitro* and *in vivo* (IL-3, IL-6, IL-11, SCF). Plasma, serum and urine from thrombocytopenic dogs and humans have been found to contain growth factors that have specific megakaryopoietic and thrombopoietic activities distinct from all known cytokines. These factors have been termed Meg-CSF, MK-CSF, megakaryocyte growth and development factor (MGDF), megakaryopoietin, and thrombopoietin but the molecular structure has not been identified until recently.

The identification of the thrombopoietic cytokine, c-mpl ligand, originated with the identification of a myeloproliferative leukemia virus (MPLV, Wend-  
ing et al., *Virology* 149:242-246 [1986]). Mice infected  
5 with this virus gave rise to multi-lineage myeloproliferation. Subsequent studies (Souyri et al., *Cell* 63:1137-1147, [1990]) demonstrated that the retrovirus encoded an oncogene (v-mpl) that when fused  
10 with viral envelope gene gave rise to a membrane anchored protein that resembles the cytoplasmic domain of the hematopoietic growth factor receptor family. V-mpl was used to probe both human and murine RNA libraries for homologous genes. Clones were identified in both species  
15 and termed c-mpl (Vigon et al., *PNAS* 89:5640-5644 [1992], Vigon et al., *Oncogene* 8:2607-2615 [1993]). C-mpl is a member of the cytokine receptor super-family with regions of homology to mIL-5rc, IL3rc, IL4rc, mEPORc and mGCSFrc. A chimera of the intracellular domain of c-mpl and the  
20 extracellular domain of hIL-4rc was transfected into a growth factor dependent cell line (BaF3). Once transfected, the cells proliferated in response to hIL4 indicating that the c-mpl cytoplasmic domain was fully sufficient to transduce a proliferative signal (Skoda et  
25 al., *EMBO J.* 12(7):2645-2653 [1993]).

Message for c-mpl was found in a number of hematopoietic cell lines using reverse transcriptase polymerase chain reaction (RT-PCR) including the  
30 pluripotent cell lines TF-1, Mo-7E, UT-7 and KU812; and erythro/megakaryocytic cell lines HEL, DAMI and K153. Transcripts were also identified in bone marrow, fetal liver, megakaryocytes, platelets and CD34+ enriched cells (Methia et al., *Blood* 82(5):1395-1401 [1993]).

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The identification of a putative receptor triggered several investigative teams to search for a natu-

rally occurring ligand for c-mpl. In June of 1994 several simultaneous publications reported on a ligand that bound to c-mpl and had megakaryocytopoietic properties (de Sauvage et al., *Nature* 369:533-539 [1994]; Lok et al., *Nature* 369:565-568 [1994]; Wendling et al., *Nature* 369:571-574 [1994] and Bartley et al., *Cell* 77:1117-1124 [1994]). The ligand referred to as c-mpl ligand, Megakaryocyte Growth and Development Factor (MGDF) or thrombopoietin (TPO) is a peptide with a predicted molecular mass of 35,000 kDa. The protein has a two domain structure with an amino-terminal domain (153 amino acids) with homology to erythropoietin and a carboxy-terminus rich in serine, threonine and proline residues which also contains several glycosylation sites. There are two potential arginine cleavage sites resulting in two shorter peptides of 25 kDa and 31 kDa forms both of which are biologically active. There is high inter-species homology between human, murine, porcine, canine, rat and rabbit c-mpl ligand and most forms are active on all species tested.

C-mpl ligand has been shown to stimulate the differentiation of CD34+ cells into cells with megakaryocyte characteristics. CD34+ cells, in the presence of c-mpl ligand, underwent endomitosis (Kaushansky et al., *Nature* 369:568-571 [1994]), expressed the megakaryocyte lineage specific cell surface antigen CD41a and had morphology characteristic of megakaryocytes. In vivo administration of c-mpl ligand gave rise to increased circulating platelets in normal mice (Lok et al., *Nature* 369:565-568 [1994]). C-mpl deficient mice generated by gene targeting demonstrated a 85% decrease in circulating platelets and megakaryocytes but had normal amounts of other hematopoietic lineages (Gurney et al., *Science* 265:1445-1447 [1994]). Absolute thrombocytopenia was not observed in these animals indicating that other cytokines may have some activity in expansion of the MK

lineage.

Studies to date show that c-mpl ligand is a cytokine with specific activity on the maturation of megakaryocytes and in platelet production. Other cytokines have been shown to have activity on megakaryocyte expansion and differentiation, including IL-3, IL-6, IL-11 and c-kit ligand. Recent studies have demonstrated that these cytokines (with the exception of IL-3) act by stimulating the production of c-mpl ligand and do not have megakaryocyte stimulating activity by themselves (Kaushansky et al., *PNAS* 92:3234-3236 [1995]).

GB 2,285,446 relates to the c-mpl ligand (thrombopoietin) and various forms of thrombopoietin which are shown to influence the replication, differentiation and maturation of megakaryocytes and megakaryocytes progenitors which may be used for the treatment of thrombocytopenia.

The ability of c-mpl ligand to stimulate the proliferation and maturation of megakaryocytes and production of platelets indicates that c-mpl ligand may have therapeutic use in restoring circulating platelets to normal amounts in those cases where the number of platelets have been reduced due to diseases or therapeutic treatments such as radiation and/or chemotherapy.

EP 675,201 A1 relates to the c-mpl ligand (Megakaryocyte growth and development factor [MGDF]), allelic variations of c-mpl ligand and c-mpl ligand attached to water soluble polymers such as polyethylene glycol.

WO 95/21920 provides the murine and human c-mpl ligand and polypeptide fragments thereof. The proteins are useful for *in vivo* and *ex vivo* therapy for stimulating platelet production.

A previously published abstract (Eaton et al., Blood 84(10) Suppl. abstract 948, [1994]) reported c-DNA for an alternative splice form of c-mpl ligand identified in man, dog and mouse. The encoded protein has 4 amino deletion at position aa112-115. Although this molecule showed no activity in their bioassays, mRNA for this variant was found to be abundant in all three species indicating that it may be a naturally occurring alternative form of c-mpl ligand. Contrary to the previously published report, we found that the 1-153  $\Delta$ 112-115 c-mpl ligand and the 1-332  $\Delta$ 112-115 c-mpl ligand were biological active.

Summary of the Invention

The present invention relates to novel c-mpl ligands of  
the following formula:

SerProAlaProProAlaCysAspLeuArgValLeuSerLysLeuLeuArgAspSer  
1                      5                      10                      15

HisValLeuHisSerArgLeuSerGlnCysProGluValHisProLeuProXaaPro  
20                      25                      30                      35

ValLeuLeuProAlaValAspXaaXaaLeuGlyGluTrpLysThrGlnMetGluGlu  
40                      45                      50                      55

ThrLysAlaGlnAspIleLeuGlyAlaValThrLeuLeuLeuGluGlyValMetAla  
60                      65                      70                      75

AlaArgGlyGlnLeuGlyProThrCysLeuSerSerLeuLeuGlyGlnLeuSerGly  
80                      85                      90                      95

GlnValArgLeuLeuLeuGlyAlaLeuGlnSerLeuLeuGlyThrGlnXaaXaaXaa  
100                      105                      110

XaaGlyArgThrThrAlaHisXaaAspProAsnAlaIlePheLeuSerPheGlnHis  
115                      120                      122                      125                      130

LeuLeuArgGlyLysValArgPheLeuMetLeuValGlyGlySerThrLeuCysVal  
135                      140                      145                      150

ArgArgAlaProProThrThrAlaValProSerArgThrSerLeuValLeuThrLeu  
155                      160                      165                      170

AsnGluLeuProAsnArgThrSerGlyLeuLeuGluThrAsnPheThrAlaSerAla  
175                      180                      185                      190

ArgThrThrGlySerGlyLeuLeuLysXaaGlnGlnGlyPheArgAlaLysIlePro



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GlyLeuLeuAsnGlnThrSerArgSerLeuAspGlnIleProGlyTyrLeuAsnArg

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IleHisGluLeuLeuAsnGlyThrArgGlyLeuPheProGlyProSerArgArgThr

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LeuGlyAlaProAspIleSerSerGlyThrSerAspThrGlySerLeuProProAsn

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265

LeuGlnProGlyTyrSerProSerProThrHisProProThrGlyGlnTyrThrLeu

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280

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PheProLeuProProThrLeuProThrProValValGlnLeuHisProLeuLeuPro

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295

300

AspProSerAlaProThrProThrProThrSerProLeuLeuAsnThrSerTyrThr

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HisSerGlnAsnLeuSerGlnGluGly

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wherein;

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Xaa at position 37 is Thr, Asp or Glu;

Xaa at position 46 is Phe, Ala, Val, Leu, Ile, Pro, Trp,  
or Met;

Xaa at position 47 is Ser, Asp or Glu;

Xaa at position 112 is deleted or Leu, Ala, Val, Ile,  
Pro, Phe, Trp, or Met;

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Xaa at position 113 is deleted or Pro, Phe, Ala, Val,  
Leu, Ile, Trp, or Met;Xaa at position 114 is deleted or Pro, Phe, Ala, Val,  
Leu, Ile, Trp, or Met;

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Xaa at position 115 is deleted or Gln, Gly, Ser, Thr,  
Tyr, or Asn;

Xaa at position 122 is Lys, Arg, His, Glu, or Asp;

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Arg  
153

wherein;

Xaa at position 37 is Thr, Asp or Glu;

Xaa at position 46 is Phe, Ala, Val, Leu, Ile, Pro, Trp,  
or Met;

5 Xaa at position 47 is Ser, Asp or Glu;

Xaa at position 112 is deleted or Leu, Ala, Val, Ile,  
Pro, Phe, Trp, or Met;

Xaa at position 113 is deleted or Pro, Phe, Ala, Val,  
Leu, Ile, Trp, or Met;

10 Xaa at position 114 is deleted or Pro, Phe, Ala, Val,  
Leu, Ile, Trp, or Met;

Xaa at position 115 is deleted or Gln, Gly, Ser, Thr,  
Tyr, or Asn;

Xaa at position 122 is Lys, Arg, His, Glu, or Asp;

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and with the proviso that at least one of the amino acids  
designated by Xaa are different from the corresponding  
amino acids of native c-mpl ligand (1-332). These c-mpl  
ligand variants may have an improved biological profile,  
20 such as increased proliferative activity and/or decreased  
side-effects, and/or improved physical properties, such  
as improved half-life, stability, and/or re-fold effi-  
ciencies.

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In addition to the use of the c-mpl ligands of the  
present invention *in vivo*, it is envisioned that *in vitro*  
uses would include the ability to stimulate bone marrow  
and blood cell activation and growth before infusion into  
patients.

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The present invention also encompasses chimera  
proteins comprising recombinant human c-mpl ligand  
muneins joined to one or more colony stimulating factor  
(CSF) including, cytokines, lymphokines, interleukins,  
35 hematopoietic growth factors (herein collectively re-  
ferred to as "colony stimulating factors") which include  
GM-CSF, CSF-1, G-CSF, M-CSF, erythropoietin (EPO), IL-1,

IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-16, LIF, flt3/flk2, human growth hormone, B-cell growth factor, B-cell differentiation factor, eosinophil differentiation factor and stem cell factor (SCF) also known as steel factor or c-kit ligand or IL-3 variant with or without a linker. These human c-mpl ligand muteins may contain amino acid substitutions, deletions and/or insertions and may also have amino acid deletions at either/or both the N- and C-termini.

This invention encompasses mixed function colony stimulating factors (chimera proteins) formed from covalently linked polypeptides, each of which may act through a different and specific cell receptor to initiate complementary biological activities. These chimeras may be characterized by having the usual activity of both of the peptides forming the chimera molecule or it may be further characterized by having a biological or physiological activity greater than simply the additive function of the presence of human c-mpl ligand or the second colony stimulating factor alone. The chimera molecule may also unexpectedly provide an enhanced effect on the activity or an activity different from that expected by the presence of human c-mpl ligand or the second colony stimulating factor. The chimera molecule may also have an improved activity profile which may include reduction of undesirable biological activities associated with native human c-mpl ligand or native cytokine.

In addition to the use of the chimera molecules of the present invention *in vivo*, it is envisioned that *in vitro* uses would include the ability to stimulate bone marrow and blood cell activation and growth before infusion into patients.

The present invention also encompasses recombinant human c-mpl ligand variant or mutant proteins (muteins)

co-administrated with one or more additional colony stimulating factors (CSF) including, cytokines, lymphokines, interleukins, hematopoietic growth factors (herein collectively referred to as "colony stimulating factors") include GM-CSF, CSF-1, G-CSF, M-CSF, erythropoietin (EPO), IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-16, LIF, flt3/flk2, human growth hormone, B-cell growth factor, B-cell differentiation factor, eosinophil differentiation factor and stem cell factor (SCF) also known as steel factor or c-kit ligand. This invention encompasses co-administered colony stimulating factors, each of which may act through a different and specific cell receptor to initiate complementary biological activities. Co-administration of c-mpl ligand and other colony stimulating factors may be characterized by having the usual activity of both of the peptides or it may be further characterized by having a biological or physiological activity greater than simply the additive function of the presence of human c-mpl ligand or the second colony stimulating factor alone. The co-administration may also unexpectedly provide an enhanced effect on the activity or an activity different from that expected by the presence of human c-mpl ligand or the second colony stimulating factor or human c-mpl ligand variant. The co-administration may also have an improved activity profile which may include reduction of undesirable biological activities associated with native human c-mpl ligand or native cytokine.

In addition to the use of co-administration of the present invention *in vivo*, it is envisioned that *in vitro* uses would include the ability to stimulate bone marrow and blood cell activation and growth before infusion into patients.

It is also envisioned that c-mpl ligands of the present invention alone, co-administered with other

colony stimulating factors, or as a component of a chimera molecule would be useful in blood banking. The c-mpl ligand would be given to a blood donor prior to giving blood to elevate their platelet count, increasing the number of platelets from each donor and thereby decreasing the cost of platelet transfusions.

Preferably the c-mpl ligand muteins of the present invention are co-administered with or comprise a chimera protein with SCF, c-kit ligand, flt3/flk2, G-CSF, IL-3 or IL-3 variant.

Most preferably the c-mpl ligand muteins of the present invention are co-administered with or comprise a chimera protein with G-CSF, or IL-3 variant.

Detailed Description of the Invention

5 Variants of c-mpl ligand of the present invention may be useful in the treatment of diseases characterized by a decreased levels of megakaryocyte cells of the hematopoietic system.

10 A variant or mutein of c-mpl ligand may be useful in the treatment or prevention of thrombocytopenia. Currently the only therapy for thrombocytopenia is platelet transfusions which are costly and carry the significant risks of infection (HIV, HBV) and alloimmunization. A variant or mutein of c-mpl ligand may alleviate or diminish the need for platelet transfusions. Severe thrombocytopenia may result from genetic defects such as Fanconi's Anemia, Wiscott-Aldrich, or May-Hegglin syndromes. Acquired thrombocytopenia may result from auto- or allo-antibodies as in Immune Thrombocytopenic Purpura, Systemic Lupus Erythromatosis, hemolytic anemia, or fetal maternal incompatibility. In addition, splenomegaly, disseminated intravascular coagulation, thrombotic thrombocytopenic purpura, infection or prosthetic heart valves may result in thrombocytopenia. Severe thrombocytopenia may also result from chemotherapy and/or radiation therapy or cancer. Thrombocytopenia may also result from marrow invasion by carcinoma, lymphoma, leukemia or fibrosis. c-mpl ligand of the present invention may be useful in the mobilization of hematopoietic progenitors and stem cells into peripheral blood. Peripheral blood derived progenitors have been shown to be effective in reconstituting patients in the setting of autologous marrow transplantation. Hematopoietic growth factors including G-CSF and GM-CSF have been shown to enhance the number of circulating progenitors and stem cells in the peripheral blood. This has simplified the procedure for peripheral stem cell collection and dramatically decreased the cost of the procedure by decreasing the

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number of plasmaphereses required. The c-mpl ligand of the present invention may be useful in mobilization of stem cells and further enhance the efficacy of peripheral stem cell transplantation. The c-mpl ligand may also be useful to increase platelet counts in platelet donors prior to apheresis to increase the number of platelets recovered from each donor.

Many drugs may cause bone marrow suppression or hematopoietic deficiencies. Examples of such drugs are AZT, DDI, alkylating agents and anti-metabolites used in chemotherapy, antibiotics such as chloramphenicol, penicillin, gancyclovir, daunomycin and sulfa drugs, phenothiazones, tranquilizers such as meprobamate, analgesics such as aminopyrine and dipyrrone, anti convulsants such as phenytoin or carbamazepine, antithyroids such as propylthiouracil and methimazole and diuretics. c-mpl ligands may be useful in preventing or treating the bone marrow suppression or hematopoietic deficiencies which often occur in patients treated with these drugs.

Hematopoietic deficiencies may also occur as a result of viral, microbial or parasitic infections and as a result of treatment for renal disease or renal failure, e.g., dialysis. c-mpl ligand may be useful in treating such hematopoietic deficiency.

The proliferation and development of stem cells and lineage-restricted progenitor cells is controlled by a large number hematopoietic growth factors or cytokines. The role of the growth factors *in vivo* is complex and incompletely understood. Some growth factors, such as Interleukin-3 (IL-3), are capable of stimulating both multipotent stem cells as well as committed progenitor cells of several lineages. Other factors, such as Erythropoietin (EPO) and c-mpl ligand, are lineage restricted. Hematopoiesis requires a complex series of cellular events in which stem cells generate continuously into



large populations of maturing cells in all major lineages. Most of these proliferative regulators can stimulate one or another type of colony formation *in vitro*, the precise pattern of colony formation stimulated by each regulator is quite distinctive. No two regulators stimulate exactly the same pattern of colony formation, as evaluated by colony numbers or, more importantly, by the lineage and maturation pattern of the cells making up the developing colonies. Proliferative responses can most readily be analyzed in simplified *in vitro* culture systems. Three quite different parameters can be distinguished: alteration in colony size, alteration in colony numbers and cell lineage. Two or more factors may act on the progenitor cell, inducing the formation of larger number of progeny thereby increasing the colony size. Two or more factors may allow increased number of progenitor cells to proliferate either because distinct subsets of progenitors cells exist that respond exclusively to one factor or because some progenitors require stimulation by two or more factors before being able to respond. Activation of additional receptors on a cell by the use of two or more factors is likely to enhance the mitotic signal because of coalescence of initially differing signal pathways into a common final pathway reaching the nucleus (Metcalf D., *Nature* 339:27, 1989). Other mechanisms could explain synergy. For example, if one signaling pathway is limited by an intermediate activation of an additional signaling pathway by a second factor may result in a superadditive response. In some cases, activation of one receptor type can induce an enhanced expression of other receptors (Metcalf D., *Blood* 82:3515-3523, 1993). Two or more factors may result in a different pattern of cell lineages than from a single factor. The use of chimera molecules comprising the c-mpl ligand of the present invention or the co-administration of the c-mpl ligands of the present invention may have the potential clinical advantage resulting from a proliferative response that is

not possible by any single factor.

Novel compounds of this invention are represented by a formula selected from the group consisting of

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$R_1-L-R_2$ ,  $R_2-L-R_1$ ,  $R_1-R_2$ ,  $R_2-R_1$ ,  $R_1-L-R_1$  and  $R_1-R_1$

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where R1 is a c-mpl ligand variant and R2 is a colony stimulating factor with a different but complementary activity. By complementary activity is meant activity which enhances or changes the response to another cell modulator. The R1 polypeptide is joined either directly or through a linker segment to the R2 polypeptide. The term "directly" defines chimeras in which the polypeptides are joined without a peptide linker. Thus L represents a chemical bound or polypeptide segment to which both R1 and R2 are joined in frame, most commonly L is a linear peptide to which R1 and R2 are bound by amide bonds linking the carboxy terminus of R1 to the amino terminus of L and carboxy terminus of L to the amino terminus of R2. By "joined in frame" is meant that there is no translation termination or disruption between the reading frames of genes encoding R1 and R2. A nonexclusive list of other growth factors, colony stimulating factors (CSFs), cytokine, lymphokine, interleukin, hematopoietic growth factor within the definition of R2, which can be joined to a c-mpl ligand variant of the present invention include GM-CSF, CSF-1, G-CSF, M-CSF, erythropoietin (EPO), IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-16, LIF, flt3/flk2, human growth hormone, B-cell growth factor, B-cell differentiation factor, eosinophil differentiation factor and stem cell factor (SCF) also known as steel factor or c-kit ligand. Additionally, this invention encompasses the use of modified R2 molecules or mutated or modified DNA sequences encoding these R2 molecules. The present invention also includes chimera molecules in which R2 is a hIL-3 variant. A "hIL-3 vari-

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ant\* is defined as a hIL-3 variant disclosed in WO 94/12639, WO 94/12638 and WO 95/00646 as well as other variants known in the art.

5 The linking group (L) is generally a polypeptide of between 1 and 500 amino acids in length. The linkers joining the two molecules are preferably designed to (1) allow the two molecules to fold and act independently of each other, (2) not have a propensity for developing an ordered secondary structure which could interfere with  
10 the functional domains of the two proteins, (3) have minimal hydrophobic or charged characteristic which could interact with the functional protein domains and (4) provide steric separation of R1 and R2 such that R1 and R2 could interact simultaneously with their corresponding  
15 receptors on a single cell. Typically surface amino acids in flexible protein regions include Gly, Asn and Ser. Virtually any permutation of amino acid sequences containing Gly, Asn and Ser would be expected to satisfy the above criteria for a linker sequence. Other neutral amino  
20 acids, such as Thr and Ala, may also be used in the linker sequence. Additional amino acids may also be included in the linkers due to the addition of unique restriction sites in the linker sequence to facilitate construction of the chimeras. More details about methods  
25 of making such chimera molecules can be found in WO 95/21254 which is herein incorporated by reference in its entirety.

30 A c-mpl ligand that has reduced activity may be useful in a chimera molecule. For example it may be advantageous to have only a small amount of megakaryocyte maturation and platelet production activity relative to the activity of the other growth factor component of the chimera molecule, such as IL-3 which stimulates  
35 multipotent stem cells as well as committed progenitor cells of several lineages including megakaryocytes. Conversely a c-mpl ligand that has increased activity

would be useful when increased megakaryocyte maturation and platelet production activity relative to the activity of the other growth factor component of the chimera molecule is desired.

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The c-mpl ligands of the present invention may also be a component of a chimera, joined to a modified mouse Fc (IgG2a) constant region (Gross A.H. et al, *J.Clin.Invest.* 95:2783-2789, [1995]). Such a chimera might be useful for purification of the c-mpl ligand, increasing protein stability and the formation of a dimer through the Fc region.

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One aspect of the present invention provides a method for selective ex-vivo expansion of stem cells. The term "stem cell" refers to the totipotent hematopoietic stem cells as well as early precursors and progenitor cells which can be isolated from bone marrow, spleen or peripheral blood. The term "expanding" refers to the differentiation and proliferation of the cells. The present invention provides a method for selective ex-vivo expansion of stem cells, comprising the steps of; (a) separating stem cells from other cells, (b) culturing said separated stem cells with a selected media which comprises a c-mpl ligand or chimera protein(s) which are in part comprised of a c-mpl ligand and (c) harvesting said stems cells. Stem cells as well as committed progenitor cells destined to become neutrophils, erythrocytes, platelets. etc., may be distinguished from most other cells by the presence or absence of particular progenitor marker antigens, such as CD34, that are present on the surface of these cells and/or by morphological characteristics. The phenotype for a highly enriched human stem cell fraction is reported as CD34+, Thy-1+ and lin-, but it is to be understood that the present invention is not limited to the expansion of this stem cell population. The CD34+ enriched human stem cell fraction can be sepa-

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rated by a number of reported methods, including affinity columns or beads, magnetic beads or flow cytometry using antibodies directed to surface antigens such as the CD34+. Further, physical separation methods such as counterflow elutriation may be used to enrich hematopoietic progenitors. The CD34+ progenitors are heterogeneous, and may be divided into several subpopulations characterized by the presence or absence of co-expression of different lineage associated cell surface associated molecules. The most immature progenitor cells do not express any known lineage-associated markers, such as HLA-DR or CD38, but they may express CD90(thy-1). Other surface antigens such as CD33, CD38, CD41, CD71, HLA-DR or c-kit can also be used to selectively isolate hematopoietic progenitors. The separated cells can be incubated in selected medium in a culture flask, sterile bag or in hollow fibers. Various colony stimulating factors may be utilized in order to selectively expand cells. Representative factors that have been utilized for ex-vivo expansion of bone marrow include, c-kit ligand, IL-3, G-CSF, GM-CSF, IL-1, IL-6, IL-11, flt-3 ligand or combinations thereof. The proliferation of the stem cells can be monitored by enumerating the number of stem cells and other cells, by standard techniques (e.g. hemacytometer, CFU, LTCIC) or by flow cytometry prior and subsequent to incubation.

Several methods for ex-vivo expansion of stem cells have been reported utilizing a number of selection methods and expansion using various colony stimulating factors including c-kit ligand (Brandt et al., *Blood* 83:1507-1514 [1994], McKenna et al., *Blood* 86:3413-3420 [1995]), IL-3 (Brandt et al., *Blood* 83:1507-1514 [1994], Sato et al., *Blood* 82:3600-3609 [1993]), G-CSF (Sato et al., *Blood* 82:3600-3609 [1993]), GM-CSF (Sato et al., *Blood* 82:3600-3609 [1993]), IL-1 (Muench et al., *Blood* 81:3463-3473 [1993]), IL-6 (Sato et al., *Blood* 8

2:3600-3609 [1993]), IL-11 (Lemoli et al., *Exp. Hem.* 21:1668-1672 [1993], Sato et al., *Blood* 82:3600-3609 [1993]), flt-3 ligand (McKenna et al., *Blood* 86:3413-3420 [1995]) and/or combinations thereof (Brandt et al., *Blood* 83:1507-1514 [1994], Haylock et al., *Blood* 80:1405-1412 [1992], Koller et al., *Biotechnology* 11:358-363 [1993], (Lemoli et al., *Exp. Hem.* 21:1668-1672 [1993]), McKenna et al., *Blood* 86:3413-3420 [1995], Muench et al., *Blood* 81:3463-3473 [1993], Patchen et al., *Biotherapy* 7:13-26 [1994], Sato et al., *Blood* 82:3600-3609 [1993], Smith et al., *Exp. Hem.* 21:870-877 [1993], Steen et al., *Stem Cells* 12:214-224 [1994], Tsujino et al., *Exp. Hem.* 21:1379-1386 [1993]). Among the individual colony stimulating factors, hIL-3 has been shown to be one of the most potent in expanding peripheral blood CD34+ cells (Sato et al., *Blood* 82:3600-3609 [1993], Kobayashi et al., *Blood* 73:1836-1841 [1989]). However, no single factor has been shown to be as effective as the combination of multiple factors. The present invention provides methods for ex vivo expansion that utilize molecules that are more effective than IL-3 alone.

Another projected clinical use of growth factors has been in the in vitro activation of hematopoietic progenitors and stem cells for gene therapy. Due to the long life-span of hematopoietic progenitor cells and the distribution of their daughter cells throughout the entire body, hematopoietic progenitor cells are good candidates for ex vivo gene transfection. In order to have the gene of interest incorporated into the genome of the hematopoietic progenitor or stem cell one needs to stimulate cell division and DNA replication. Hematopoietic stem cells cycle at a very low frequency which means that growth factors may be useful to promote gene transduction and thereby enhance the clinical prospects for gene therapy. Potential applications of gene therapy (review Crystal, *Science* 270:404-410 [1995]) include; 1)

the treatment of many congenital metabolic disorders and immunodeficiencies (Kay and Woo, *Trends Genet.* 10:253-257 [1994]), 2) neurological disorders (Freedmann, *Trends Genet.* 10:210-214 [1994]), 3) cancer (Culver and Blaese, *Trends Genet.* 10:174-178 [1994]) and 4) infectious diseases (Gilboa and Smith, *Trends Genet.* 10:139-143 [1994]).

There are a variety of methods, known to those with skill in the art, for introducing genetic material into a host cell. A number of vectors, both viral and non-viral have been developed for transferring therapeutic genes into primary cells. Viral based vectors include; 1) replication-deficient recombinant retrovirus (Boris-Lawrie and Temin, *Curr. Opin. Genet. Dev.* 3:102-109 [1993], Boris-Lawrie and Temin, *Annal. New York Acad. Sci.* 716:59-71 [1994], Miller, *Current Top. Microbiol. Immunol.* 158:1-24 [1992]) and replication-deficient recombinant adenovirus (Berkner, *BioTechniques* 6:616-629 [1988], Berkner, *Current Top. Microbiol. Immunol.* 158:39-66 [1992], Brody and Crystal, *Annal. New York Acad. Sci.* 716:90-103 [1994]). Non-viral based vectors include protein/DNA complexes (Cristiano et al., *PNAS USA.* 90:2122-2126 [1993], Curiel et al., *PNAS USA* 88:8850-8854 [1991], Curiel, *Annal. New York Acad. Sci.* 716:36-58 [1994]), electroporation and liposome mediated delivery such as cationic liposomes (Farhood et al., *Annal. New York Acad. Sci.* 716:23-35 [1994]).

The present invention provides an improvement to the existing methods of expanding hematopoietic cells, which new genetic material has been introduced, in that it provides methods utilizing chimera proteins that have improved biological, including an activity not seen by any single colony stimulation factor and/or physical properties.

The present invention also provides genes, encoding c-mpl ligands and chimera proteins, which can be introduced

into cells for gene therapy.

As another aspect of the present invention, there is provided a novel method for producing the novel family of human c-mpl ligand muteins. The method of the present invention involves culturing a suitable cell or cell line, which has been transformed with a vector containing a DNA sequence coding for expression of a novel c-mpl ligand mutant polypeptide. Suitable cells or cell lines may be bacterial cells. For example, the various strains of *E. coli* are well-known as host cells in the field of biotechnology. Examples of such strains include *E. coli* strains JM101 (Yanisch-Perron, et al., *Gene*, 33: 103-119 [1985]) and MON105 (Obukowicz, et al., *Appl. and Envir. Micr.*, 58: 1511-1523 [1992]). Various strains of *B. subtilis* may also be employed in this method. Many strains of yeast cells known to those skilled in the art are also available as host cells for expression of the polypeptides of the present invention.

Also suitable for use in the present invention are mammalian cells, such as Chinese hamster ovary cells (CHO). General methods for expression of foreign genes in mammalian cells are reviewed in: Kaufman, R. J. (1987) High level production of proteins in mammalian cells, in Genetic Engineering, Principles and Methods, Vol. 9, J. K. Setlow, editor, Plenum Press, New York. An expression vector is constructed in which a strong promoter capable of functioning in mammalian cells drives transcription of a eukaryotic secretion signal peptide coding region, which is translationally joined to the coding region for the c-mpl ligand variant. For example, plasmids such as pCDNA I/Neo, pRc/RSV, and pRc/CMV (obtained from Invitrogen Corp., San Diego, California) can be used. After construction of the vector containing the c-mpl ligand gene, the vector DNA is transfected into mammalian cells. Such cells can be, for example, the COS7, HeLa,



BHK, CHO, or mouse L lines. The cells can be cultured, for example, in DMEM media (JRH Scientific). The c-mpl ligand secreted into the media can be recovered by standard biochemical approaches following transient expression 24 - 72 hours after transfection of the cells or after establishment of stable cell lines following selection for neomycin resistance. The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. See, e.g., Gething and Sambrook, *Nature* 293:620-625 [1981], or alternatively, Kaufman et al., *Mol. Cell. Biol.* 5 (7):1750-1759 [1985] or Howley et al., U.S. Pat. No. 4,419,446. Another suitable mammalian cell line is the monkey COS-1 cell line. A similarly useful mammalian cell line is the CV-1 cell line.

Where desired, insect cells may be utilized as host cells in the method of the present invention. See, e.g. Miller et al, *Genetic Engineering*, 8:277-298 (Plenum Press 1986) and references cited therein. In addition, general methods for expression of foreign genes in insect cells using Baculovirus vectors are described in: Summers, M. D. and Smith, G. E. (1987) - A manual of methods for Baculovirus vectors and insect cell culture procedures, Texas Agricultural Experiment Station Bulletin No. 1555. An expression vector is constructed comprising a Baculovirus transfer vector, in which a strong Baculovirus promoter (such as the polyhedron promoter) drives transcription of a eukaryotic secretion signal peptide coding region, which is translationally joined to the coding region for the c-mpl ligand polypeptide. For example, the plasmid pVL1392 (obtained from Invitrogen Corp., San Diego, California) can be used. After construction of the vector carrying the c-mpl ligand gene, two micrograms of this DNA is cotransfected with one microgram of Baculovirus DNA (see Summers & Smith, 1987) into insect cells, strain SF9. Pure recombinant

Baculovirus carrying the hIL-3 variant is used to infect cells cultured, for example, in Excell 401 serum-free medium (JRH Biosciences, Lenexa, Kansas). The c-mpl ligand secreted into the medium can be recovered by standard biochemical approaches.

Another aspect of the present invention provides plasmid DNA vectors for use in the method of expression of these novel c-mpl ligand muteins. These vectors contain the novel DNA sequences described above which code for the novel polypeptides of the invention. Appropriate vectors which can transform microorganisms capable of expressing the c-mpl ligand muteins include expression vectors comprising nucleotide sequences coding for the c-mpl ligand muteins joined to transcriptional and translational regulatory sequences which are selected according to the host cells used.

Vectors incorporating modified sequences as described above are included in the present invention and are useful in the production of the c-mpl ligand mutant polypeptides. The vector employed in the method also contains selected regulatory sequences in operative association with the DNA coding sequences of the invention and capable of directing the replication and expression thereof in selected host cells.

Other aspects of the present invention are methods and therapeutic compositions for treating the conditions referred to above. Such compositions comprise a therapeutically effective amount of one or more of the c-mpl ligand muteins of the present invention in a mixture with a pharmaceutically acceptable carrier. This composition can be administered either parenterally, intravenously or subcutaneously. When administered, the therapeutic composition for use in this invention is preferably in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such a parenterally acceptable protein solution, having due regard to pH, isotonicity, stability and the like, is within the skill

of the art.

The dosage regimen involved in a method for treating the above-described conditions will be determined by the attending physician considering various factors which modify the action of drugs, e.g. the condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. Generally, a daily regimen may be in the range of 0.1 - 100  $\mu\text{g/kg}$  of non-glycosylated c-mpl ligand protein per kilogram of body weight. Dosages would be adjusted relative to the activity of a given mutein and it would not be unreasonable to note that dosage regimens may include doses as low as 0.1 microgram and as high as 1 milligram per kilogram of body weight per day. In addition, there may exist specific circumstances where dosages of c-mpl ligand mutein would be adjusted higher or lower than the range of 0.1 - 100 micrograms per kilogram of body weight. These include co-administration with other CSF or growth factors; co-administration with chemotherapeutic drugs and/or radiation; the use of glycosylated c-mpl ligand mutein; and various patient-related issues mentioned earlier in this section. As indicated above, the therapeutic method and compositions may also include co-administration with other human factors. A non-exclusive list of other appropriate hematopoietins, CSFs and interleukins for simultaneous or serial co-administration or chimera with the polypeptides of the present invention includes GM-CSF, CSF-1, G-CSF, Meg-CSF, M-CSF, erythropoietin (EPO), IL-1, IL-3, IL-4, IL-2, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-16, LIF, flt2/flk3 ligand, B-cell growth factor, B-cell differentiation factor and eosinophil differentiation factor, stem cell factor (SCF) also known as steel factor or c-kit ligand (herein collectively referred to as "colony stimulating factors"), or combinations thereof. In addition to the list above, IL-3

variants taught in WO 94/12638, WO 94/12639 and  
WO 95/00646 can be co-administered or joined as a chimera  
with the c-mpl ligand polypeptides of the present inven-  
tion. The c-mpl ligands of the present invention can be  
5 joined or co-administered as with another "colony stimu-  
lating factor" as discussed above in a fashion taught in  
WO 95/20977 and WO 95/21254. The dosage recited above  
would be adjusted to compensate for such additional  
components in the therapeutic composition. Progress of  
10 the treated patient can be monitored by periodic assess-  
ment of the hematological profile, e.g., differential  
cell count and the like.

All references, patents or applications cited herein  
15 are incorporated by reference in their entirety.

#### **Recombinant DNA methods**

Unless noted otherwise, all specialty chemicals were  
obtained from Sigma Co., (St. Louis, MO). Restriction  
20 endonucleases and T4 DNA-ligase were obtained from New  
England Biolabs (Beverly, MA).

#### **Reverse transcriptase/polymerase chain reaction**

The alternate forms of c-mpl ligand can be isolated  
25 using reverse transcriptase/polymerase chain reaction  
(RT/PCR) technology. Synthetic primers are designed so  
that they would anneal to either c-mpl ligand DNA or mRNA  
(c-mpl ligand sequence based on Genebank accession  
#L33410 or de Sauvage et al., Nature 369: 533-538 [1994])  
30 for priming first-strand complementary DNA (cDNA) synthe-  
sis. The resulting cDNA is used as a template in PCR  
(Saiki, 1985) to generate double-stranded DNA (dsDNA or  
DNA) which can be used in additional PCR or digested with  
appropriate restriction enzymes for transfer to  
35 Baculovirus, mammalian or bacterial, such as *E. coli*,  
expression plasmids.

For the reverse transcriptase (RT) reaction, human fetal

(lot #38130) and adult liver (lot #46018) A+ RNA can be obtained from Clontech (Palo Alto, CA). The RT reactions are carried out using a cDNA Cycle™ Kit obtained from Invitrogen (San Diego, CA). One microgram (ug) of each RNA sample is denatured at 65°C for 10 min. in the presence of either random primers, oligo dT primer or a specific 3' anti-sense primer. Following denaturation, the samples are cooled for 2 min. on ice and spun down for 10 sec. at 10,000 x g. RNase inhibitor, reverse transcriptase buffer, deoxynucleotides, sodium pyrophosphate and reverse transcriptase are added as described by manufacturer, and the 20 microliter reaction is incubated at 42°C for 1 hr.

For PCR specific 5' sense and 3' anti-sense primers are added to the RT reactions and the PCR is carried out using reagents from Boehringer Mannheim (Indianapolis, IN) or Perkin-Elmer (Norwalk, CT) as described by the manufacturers using Taq polymerase. The PCR reactions are subjected to 30 cycles of the following; 1 min. @ 94°C, 1 min. @ 58°C, 90 sec. @ 72°C. An equal volume of loading dye (0.01% each bromophenol blue and xylene cyanole blue) is added to 10 microliters of the final product for electrophoresis through a 1% SeaKem® LE agarose (FMC, Rockland, ME) gel in the presence of 1x TBE/EtBr (Tris-borate-EDTA plus ethidium bromide; Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, [1989]). For molecular weight standards, 1 microgram of phiX174 phage DNA digested with HaeIII restriction enzyme (New England Biolabs, Beverly, MA) is loaded onto the gel. The product (about 1090 base pairs) is visualized using a short-wave UV light source. The reactions are purified using a Wizard™ PCR Preps kit from Promega (Madison, WI). Briefly, the PCR reactions are added to 100 microliters of Direct Purification buffer, and 1 milliliter (mL) of PCR Preps DNA Purification Resin is added to this mixture. After 1 minute incubation at

24°C, the supernatant is removed by vacuum filtration through a filtration column. Two mLs of 80% isopropanol is used to wash the resin via vacuum filtration. The column containing the resin is then subjected to centrifugation at 10,000 x g for 30 seconds to remove residual isopropanol. The PCR product is eluted with 50 microliters of 10 mM Tris-HCl, 1 mM EDTA, pH7.4, via centrifugation at 10,000 x g for 30 seconds followed by transfer of supernatant to a new tube.

#### Subcloning c-mpl ligand forms into expression vectors

The c-mpl ligand PCR products are digested with the appropriate restriction enzymes for ligation to either baculovirus, mammalian or *E. coli* expression vectors. The mammalian expression vectors are derivatives of pMON3359 which is a pUC18-based vector containing a mammalian expression cassette. The cassette includes a herpes simplex viral promoter IE110 (-800 to +120) and a SV40 late poly-adenylation (poly-A) signal which has been subcloned into the pUC18 polylinker (See Hippenmeyer et al., *Bio/Technology* :1037-1041 [1993]). Restriction enzyme digestions are incubated for 1 hour at 37°C as described by the manufacturer prior to electrophoresis through a 1% agarose/1X TBE/EtBr gel. Fragments are first visualized by long-wave UV and gel-purified using a Qiaex DNA Extraction kit (Qiagen, Chatsworth, CA). The DNA fragments are purified from the resin by agarose solubilization, addition of a DNA-binding resin, and extensive washing of the resin prior to elution with water. The purified DNA products are combined at a molar excess of PCR product to vector and the ligation reactions are carried out according to the manufacturer's recommended conditions for T4 DNA ligase. The *E. coli* expression vectors that direct high-level production of heterologous proteins in the cytoplasm are derivatives of that described elsewhere (Olins et al., *Methods Enzym.*, 185: 115-119 [1988] and Rangwala et al.,

Gene, 122: 263-269 [1992]). The expression cassette consists of the *recA* promoter and T7 gene 10 ribosome binding site (RBS) as well as an M13 origin of replication or a tandem inverted repeat of a phage P22 gene which acts as a transcription terminator. These cassettes are on a plasmid with the pBR327 origin of replication and encode a gene either for spectinomycin or ampicillin resistance.

#### Transformation of *E. coli* strains

*E. coli* strains such as TG1 (Amersham Corp., Arlington Heights, IL) JM101 (Yanish-Perron C., et al. Gene 33: 103-119 [1985]), or DH5 $\alpha$  (Life Technologies, Gaithersburg, MD) are used for transformation of ligation reactions and are the source of plasmid DNA for transfecting mammalian cells. *E. coli* strains MON105 and JM101 can be obtained from the American Type Culture Collection (ATCC, Rockville, MD) and are hosts for expressing alternate forms of c-mpl ligand in the cytoplasm and periplasmic space of *E. coli*.

MON105 ATCC#55204: F<sup>-</sup>, lambda<sup>-</sup>, IN(rrnD, rrE)1, rpoD<sup>+</sup>, rpoH358

JM101 ATCC#33876: delta (pro lac), supE, thi, F'(traD36, proA<sup>+</sup>B<sup>+</sup>, lacI<sup>q</sup>, lacZdeltaM15)

TG1: delta (lac-pro), supE, thi, hsdD5/F'(traD36, proA<sup>+</sup>B<sup>+</sup>, lacI<sup>q</sup>, lacZdeltaM15)

DH5 $\alpha$ : F<sup>-</sup>  $\Phi$ 80dlacZM15  $\Delta$ (lacZYA-argF)U169 deoR recA1 endA1 hsdR17(r<sub>K</sub><sup>-</sup>, m<sub>K</sub><sup>+</sup>) supE44  $\lambda$ <sup>-</sup> thi-1 gyrA96 relA1

*E. coli* strains can be rendered competent to take up DNA using a CaCl<sub>2</sub> method. Typically, 20 to 50 mLs of cells are grown in LB medium (1% bacto-tryptone, 0.5% bacto-

yeast extract, 150 millimolar NaCl) to a density of approximately 1.0 optical density units at 600 nanometers (OD<sub>600</sub>) as measured by a Baush & Lomb Spectronic spectrophotometer (Rochester, NY). The cells are collected by centrifugation and resuspended in one-fifth culture volume of CaCl<sub>2</sub> solution (50 millimolar CaCl<sub>2</sub>, 10 millimolar Tris-HCl, pH7.4) and are held at 4°C for 30 minutes. The cells are again collected by centrifugation and resuspended in one tenth culture volume of CaCl<sub>2</sub> solution. Ligated DNA is added to 0.2 mL of these cells, and the samples are held at 4°C for 1 hour. The samples are shifted to 42°C for two minutes and 1.0 mL of LB is added prior to shaking the samples at 37°C for one hour. Cells from these samples are spread on plates (LB medium plus 1.5% bacto-agar) containing either ampicillin (100 micrograms/mL, ug/mL) when selecting for ampicillin-resistant transformants, or spectinomycin (75 ug/mL) when selecting for spectinomycin-resistant transformants. The plates are incubated overnight at 37°C. Single colonies are picked, grown in LB supplemented with appropriate antibiotic for 6-16 hours at 37°C with shaking. Colonies are picked and inoculated into LB plus appropriate antibiotic (100 ug/mL ampicillin or 75 ug/mL spectinomycin) and are grown at 37°C while shaking. Before harvesting the cultures, 1 ul of cells are analyzed by PCR for the presence of a c-mpl ligand gene. The PCR is carried out using a combination of primers that anneal to the c-mpl ligand gene and/or vector. After the PCR is complete, loading dye is added to the sample followed by electrophoresis as described earlier. A gene has been ligated to the vector when a PCR product of the expected size is observed.

#### DNA isolation and characterization

Plasmid DNA is isolated using the Promega Wizard™ Miniprep kit (Madison, WI) or the Qiagen QIAwell Plasmid



isolation kits (Chatsworth, CA). Both kits follow the same general procedure for plasmid DNA isolation. Briefly, cells are pelleted by centrifugation (5000 x g), plasmid DNA released with sequential NaOH & acid treatment, and cellular debris is removed by centrifugation (10000 x g). The supernatant (containing the plasmid DNA) is loaded onto a column containing a DNA-binding resin, the column is washed, and plasmid DNA eluted with TE. Between 0.2 and 1.0 ug of plasmid DNA from the truncated c-mpl ligand clones is digested with appropriate restriction enzymes followed by electrophoresis as described earlier to confirm the presence of a c-mpl gene fragment released from the vector. *E. coli* harboring the desired plasmid DNA are inoculated into 100 mls of LB plus appropriate antibiotic for overnight growth at 37°C in an air incubator while shaking. Plasmid DNA is isolated using the Qiagen Plasmid Midi kit (Chatsworth, CA) which is a scaled-up version of the Qiagen QIAwell Plasmid isolation kit described earlier. The DNA is used for DNA sequencing, further restriction enzyme digestion, additional subcloning of DNA fragments and transfection into mammalian or *E. coli* cells.

Purified recombinant double-stranded DNA is sequenced using either the Applied Biosystems Inc. (ABI, Foster City, CA) PRISM™ Ready Reaction DyeDeoxy™ Terminator Sequencing system or United States Biochemical (Cleveland, OH) Sequenase™ Version 2.0 DNA Sequencing kit. The ABI system relies on incorporation of four fluorescence labelled dideoxy nucleotides into single-stranded DNA during multiple rounds of amplification. Plasmid DNA and a sequencing primer are added to the reaction mixture (including Taq DNA polymerase, buffer and nucleotides), which is subjected to 25 cycles of amplification (30 seconds at 96°C, 15 seconds at 50°C, 4 minutes at 60°C). Following amplification, unincorporated nucleotides are removed using Centri-Sep spin columns (equilibrated in water) as described by Princeton Separ-

tions, Inc. (Adelphia, NJ). Briefly, the samples are loaded onto a column that has excess water removed by centrifugation (700 x g) for 2 minutes, and the purified sequencing product is eluted by centrifugation (700 x g) for 4 minutes. The samples are then dried down in a Speed Vac (Savant, Hicksville, NY) prior to addition of loading solution. The samples are electrophoresed through a 4.75% polyacrylamide sequencing gel containing 7M urea in 1X TBE at 70 watts constant power. The ABI system uses a detector that recognizes each differentially labelled PCR product as they are being subjected to electrophoresis.

For the Sequenase™ sequencing system, plasmid DNA is denatured with NaOH and neutralized with ammonium hydroxide (as described by manufacturer) to provide single-stranded DNA for sequencing. After annealing a sequencing primer to the denatured DNA for 30 minutes in the presence of buffer, alpha[<sup>33</sup>P]deoxy adenosine triphosphate, DTT, deoxy and dideoxy nucleotides are added. After 5 minutes at room temperature, the reaction is split into four tubes, each of which contains additional deoxy nucleotides as well as one type of dideoxy nucleotide per tube. After 5 minutes at 37°C, the reactions are terminated with loading dye. The samples are heated to 80°C for 2 minutes and subjected to electrophoresis on a 6.0% polyacrylamide sequencing gel containing 7M urea in 1X TBE at 70 watts constant power. The gel is fixed in 10% acetic acid for 30 minutes and dried under a vacuum at 80° C for 30 minutes. The dried gel is placed next to X ray film overnight at room temperature and the film is developed using an Eastman Kodak X-OMAT M20 processor (Rochester, NY).

#### **Production of novel forms of c-mpl ligand**

#### **Mammalian Cell Transfection/Production of Conditioned**

### Media

The BHK-21 cell line can be obtained from the ATCC (Rockville, MD). The cells are cultured in Dulbecco's modified Eagle media (DMEM/high-glucose), supplemented to 2 millimolar (mM) L-glutamine and 10% fetal bovine serum (FBS). This formulation is designated BHK growth media. Selective media is BHK growth media supplemented with 453 units/ml hygromycin B (Calbiochem, San Diego, CA). The BHK-21 cell line was previously stably transfected with the HSV transactivating protein VP16, which transactivates the IE110 promoter found on the plasmid pMON3359 (See Hippenmeyer et al., *Bio/Technology* :1037-1041 [1993]). The VP16 protein drives expression of genes inserted behind the IE110 promoter. BHK-21 cells expressing the transactivating protein VP16 is designated BHK-VP16. The plasmid pMON1118 (See Highkin et al., *Poultry Sci.* 70: 970-981 [1991]) expresses the hygromycin resistance gene from the SV40 promoter. A similar plasmid is available from ATCC, pSV2-hph.

BHK-VP16 cells are seeded into a 60 millimeter (mm) tissue culture dish at  $3 \times 10^5$  cells per dish 24 hours prior to transfection. Cells are transfected for 16 hours in 3 mL of "OPTIMEM"™ (Gibco-BRL, Gaithersburg, MD) containing 10 ug of plasmid DNA containing the gene of interest, 3 ug hygromycin resistance plasmid, pMON1118, and 80 ug of "LIPOFECTAMINE"™ (Gibco-BRL) per dish. The media is subsequently aspirated and replaced with 3 mL of growth media. At 48 hours post-transfection, media from each dish is collected and assayed for activity (transient conditioned media). The cells are removed from the dish by trypsin-EDTA, diluted 1:10 and transferred to 100 mm tissue culture dishes containing 10 mL of selective media. After approximately 7 days in selective media, resistant cells grow into colonies several millimeters in diameter. The colonies were removed from the dish with filter paper (cut to approximately the same size as the colonies and soaked in trypsin/EDTA) and transferred to

individual wells of a 24 well plate containing 1 mL of selective media. After the clones are grown to confluence, the conditioned media is reassayed, and positive clones are expanded into growth media.

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Expression and purification of recombinant c-mpl ligand proteins from *E. coli*

*E. coli* strain MON105 harboring the plasmid of interest are grown at 37°C in M9 plus casamino acids medium with shaking in a air incubator Model G25 from New Brunswick Scientific (Edison, New Jersey). Growth is monitored at OD<sub>600</sub> until it reaches a value of 1.0 at which time Nalidixic acid (10 milligrams/mL) in 0.1 N NaOH is added to a final concentration of 50 µg/mL. The cultures are then shaken at 37°C for three to four additional hours. A high degree of aeration is maintained throughout culture period in order to achieve maximal production of the desired gene product. The cells are examined under a light microscope for the presence of inclusion bodies (IB). One mL aliquots of the culture are removed for analysis of protein content by boiling the pelleted cells, treating them with reducing buffer and electrophoresis via SDS-PAGE (see Maniatis et al. *Molecular Cloning: A Laboratory Manual*, [1982]).

After centrifugation (5000 x g) to pellet the cells, the first step in purification of the protein is either sonication or homogenization of the cells. For sonication, the cells are resuspended in one-tenth volume (based on culture size) sonication buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). These resuspended cells are subjected to several repeated sonication bursts using the microtip from a Sonicator cell disrupter, Model W-375 obtained from Heat Systems-Ultrasonics Inc. (Farmingdale, New York). The extent of sonication is monitored by examining the homogenates under a light microscope.

After all of the cells are disrupted, the homogenates are fractionated by centrifugation at 10000 x g for 20 min-

utes at 4°C in a JA-20 rotor and J2-21 centrifuge (Beckman, Fullerton, CA). Alternatively, the IBs are released from the cells by lysing the cells in sonication buffer with a Manton-Gaulin homogenizer (Holland) followed by centrifugation as above. The IB pellets, which are highly enriched for the recombinant protein, are then subjected to another round of sonication and centrifugation as described above. The recombinant protein is purified by a variety of standard methods. The most common methods involve solubilization of the IBs with 4-6 molar urea or guanidine-HCl buffers at pH 9-12, and air oxidation/folding in the presence of catalytic concentrations of cysteine, beta-mercaptoethanol or dithiothreitol for 24 to 72 hours. The protein is purified from *E. coli* contaminants using ion-exchange chromatography, such as Q-sepharose (anion) and S-sepharose (cation), gel filtration, hydrophobic chromatography or reversed phase HPLC. After dialysis against a low ionic strength buffer, the purified protein is stored frozen or lyophilized.

Additional details about recombinant DNA methods which may be used to create the variants and chimera proteins, express them in bacteria, mammalian cells or insect cells, purification and refold of the desired proteins and assays for determining the bioactivity of the proteins may be found in WO 95/00646, WO 94/12639, WO 94/12638, WO 95/20976, WO 95/21197, WO 95/20977, and WO 95/21254 which are hereby incorporated by reference in their entirety.

Further details known to those skilled in the art may be found in T. Maniatis, et al., Molecular Cloning. A Laboratory Manual, Cold Spring Harbor Laboratory (1982) and references cited therein, incorporated herein by reference; and in J. Sambrook, et al., Molecular Cloning. A Laboratory Manual, 2nd edition, Cold Spring Harbor

Laboratory (1989) and references cited therein, incorporated herein by reference.

5 Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following preferred specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in  
10 any way whatsoever.

#### Example 1

##### Isolation of c-mpl ligand gene products

15 A. Reverse transcriptase reaction  
Human fetal (lot #38130) and adult liver (lot #46018) A+ RNA were obtained from Clontech (Palo Alto, CA). The first strand cDNA reactions were carried out using a cDNA Cycle™ Kit obtained from Invitrogen (San Diego, CA). In  
20 the first reverse transcriptase reaction (RT#1) random primers (supplied in cDNA Cycle™ Kit) were used. In the second reverse transcriptase reaction (RT#2) oligo dT primer (supplied in cDNA Cycle™ Kit) and the specific 3' anti-sense primer, c-mplEcoRI [SEQ ID NO:23] were used.  
25 The c-mplEcoRI primer anneals to the 3' end of the c-mpl ligand gene coding sequence (bases #1257-1278 based on c-mpl ligand sequence from Genbank accession #L33410 or de Sauvage et al., Nature 369: 533-538 [1994]) and encodes an EcoRI restriction enzyme site 3' to the termination  
30 codon.

##### B. Polymerase chain reaction

###### 1. 1-332 c-mpl ligand

35 For amplification of 1-332 amino acid c-mpl ligand gene fragments, the product of reverse transcriptase reactions RT#1 and RT#2 were used as templates in PCR. In the first polymerase chain reaction (PCR#1), RT#1 served as the

template and the primers c-mplEcoRI [SEQ ID NO:23] and the 5' sense primer c-mplBglII [SEQ ID NO:24] were added to the reaction. The c-mplBglII [SEQ ID NO:24] primer anneals to the 5' end of the coding sequence for the c-mpl ligand gene (bases #207-230) and encodes a BglII restriction enzyme site 5' to the initiator methionine codon. In (PCR#2), RT#2 served as the template and only the c-mplBglII [SEQ ID NO:24] primer was added.

## 2. 1-153 c-mpl ligand

For amplification of 1-153 amino acid c-mpl ligand gene fragments, PCR#2 served as the template for amplification with a combination of the following primers; c-mplNcoI [SEQ ID NO:25], c-mplHindIII [SEQ ID NO:26], Ecocmpl [SEQ ID NO:29]

In polymerase chain reaction #3 (PCR #3), for generation of a 1-153 c-mpl ligand with a translation termination codon following amino acid #153, the c-mplNcoI [SEQ ID NO:25] and c-mplHindIII [SEQ ID NO:26] primers were used. The c-mplNcoI primer [SEQ ID NO:25] anneals to the c-mpl ligand gene (bases #279-311) resulting in codon choice degeneracy so that the gene could be efficiently transcribed and translated in *Escherichia coli* (*E. coli*). Transcription and translation of foreign genes in *E. coli* is affected by codon choice at the 5' end of the gene, and *E. coli*-preferred codons (See Chen et al., *DNA* :365-374 [1982]) usually lead to higher levels of expression. By providing multiple choices in codon sequence, multiple clones can be screened for high level expression. The NcoI restriction enzyme site added to the 5' end of the gene codes for methionine and alanine codons prior to the serine which is referred here as c-mpl ligand amino acid #1. The c-mplHindIII [SEQ ID NO:26] adds both a termination codon and a HindIII restriction enzyme site immediately following the final codon, arginine, which is referred to herein as amino acid #153. In polymerase

chain reaction #4 (PCR#4), for generation of a 1-153 c-mpl ligand without a termination codon following amino acid #153, the c-mplNcoI [SEQ ID NO:25] and Ecocmpl [SEQ ID NO:29] primers were used with PCR#2 as the template. The Ecocmpl [SEQ ID NO:29] primer encodes a EcoRI site (GAATTC) in-frame with the c-mpl ligand gene, which create a glutamate and phenylalanine codons. These PCR reactions were designed so that the product resulted in the sequence from bases #279-737, encoding amino acids #1-153, which could be transferred into multiple expression systems.

#### Example 2

15

#### BHK expression plasmid for 1-332 c-mpl ligand gene products

The full length c-mpl ligand PCR products (PCR#1 and PCR#2) were digested with BglII and EcoRI restriction enzymes and combined for transfer to a mammalian expression vector. The expression vector, pMON3976, was digested with BamHI and EcoRI (ca. 3750 bp), which allowed it to accept the BglII-EcoRI PCR fragments (ca. 1050 bp). pMON3976 is a derivative of pMON3359 which is a pUC18-based vector containing a mammalian expression cassette. The cassette includes the herpes simplex viral promoter IE110 (-800 to +120) and a SV40 late poly-adenylation (poly-A) signal subcloned into the pUC18 polylinker (See Hippenmeyer et al., *Bio/Technology* :1037-1041 [1993]). The original EcoRI site 5' to the promoter had been removed and a new EcoRI site added 3' to the BamHI site. These unique restriction enzyme sites are located between the promoter and poly-A signal to facilitate subcloning DNA fragments as BamHI-EcoRI or BglII-EcoRI fragments in a 5' to 3' direction for transcription and translation. The BglII site (5' end of the gene) is destroyed when ligated to the BamHI site of the vector. Plasmid DNA was



prepared from individual clones and the c-mpl ligand insert portion were sequenced. One of the clones identified, pMON26451-3, encodes amino acids 1-332 c-mpl ligand with a deletion of amino acids #112-115. The plasmid, pMON26451-3, contains the DNA sequence [SEQ ID NO:39] which encodes the polypeptide represented by [SEQ ID NO:37]. One of the clones identified, pMON26451-1, encodes amino acids 1-332 c-mpl ligand with a amino acid substitution K(122)E. The plasmid, pMON26451-1, contains the DNA sequence [SEQ ID NO:59] which encodes the polypeptide represented by [SEQ ID NO:66]. One of the clones identified, pMON26451-4, encodes amino acids 1-332 c-mpl ligand with two amino acid substitutions P(46)L and W(200)R. The plasmid, pMON26451-4, contains the DNA sequence [SEQ ID NO:60] which encodes the polypeptide represented by [SEQ ID NO:67].

### Example 3

#### BHK expression plasmid for 1-153 c-mpl ligand gene products

The 1-153 c-mpl ligand PCR product (PCR#3) was digested with NcoI and HindIII restriction enzymes (ca. 460 bp) for transfer to a mammalian expression vector. The expression vector, pMON3934, was digested with NcoI and HindIII (ca. 3800 bp). pMON3934 is a derivative of pMON3359 which is a pUC18-based vector containing a mammalian expression cassette. The cassette includes the herpes simplex viral promoter IE110 (-800 to +120), a modified human IL-3 signal peptide sequence and a SV40 late poly-adenylation (poly-A) signal subcloned into the pUC18 polylinker (See Hippenmeyer et al., *Bio/Technology* :1037-1041 [1993]). The human IL-3 signal peptide sequence, which had been subcloned as a BamHI fragment into the unique BamHI site between the IE110 promoter and poly-A signal, contains an NcoI site at its 3' end fol-

lowed by a HindIII site. This cloning also leaves a BamHI site 5' to the signal peptide and another BamHI site 3' to the HindIII site. When an NcoI-HindIII fragment is subcloned into pMON3934, the DNA sequence between the NcoI and HindIII sites is lost. This expression cassette is useful for secretion of proteins outside the cell. The DNA sequence of the signal peptide is shown below (restriction enzyme sites are indicated above). The ATG (methionine) codon within the NcoI site is in-frame with the initiator ATG of the signal peptide (underlined);

BamHI NcoI

5' GGATCCACCATGAGCCGCCTGCCCGTCCTGCTCCTGCTCCAACCTCGTCCGCCCGCCCATGG  
[SEQ ID NO:27]

MetSerArgLeuProValLeuLeuLeuLeuGlnLeuLeuValArgProAlaMet  
[SEQ ID NO:28]

Plasmid DNA was prepared from individual clones and the c-mpl ligand insert portion were sequenced. One of the clones identified, pMON26448, encodes amino acids 1-153 c-mpl ligand. The plasmid, pMON26448, contains the DNA sequence [SEQ ID NO:58] which encodes the polypeptide represented by [SEQ ID NO:65]. One of the clones identified, pMON26450, encodes amino acids 1-153 c-mpl ligand with a deletion of amino acids #112-115. The plasmid, pMON26450, contains the DNA sequence [SEQ ID NO:40] which encodes the polypeptide represented by [SEQ ID NO:38].

#### Example 4

##### E. coli expression plasmid for 1-153 A112-115 c-mpl ligand gene product

The 1-153 c-mpl ligand PCR products (PCR#3) were digested with NcoI and HindIII restriction enzymes (ca. 460 bp) for transfer to an E. coli expression vector, pMON3935, digested with NcoI and HindIII (ca. 3250 bp). pMON3935 directs high-level production of heterologous proteins in

the cytoplasm. The expression cassette of pMON3935 consists of the recA promoter and T7 gene 10 ribosome binding site (RBS) described elsewhere (Olins et al., *Methods Enzym.*, 185: 115-119 [1988]) as well as a tandem inverted repeat of a phage P22 gene which functions as a transcription terminator. The cassette is on a plasmid that contains the pBR327 origin of replication and a gene that encodes for spectinomycin resistance. The NcoI restriction enzyme site follows the gene 10 RBS, and the HindIII restriction enzyme site is located between the NcoI site and the P22 terminator. Several different clones were screened for expression of a unique 17 Kd protein via SDS-PAGE as described earlier. pMON26453, encoding amino acids 1-153 c-mpl ligand with a deletion of amino acids #112-115, is a result of this cloning event. The plasmid, pMON26453, contains the DNA sequence [SEQ ID NO:49] which encodes the polypeptide represented by [SEQ ID NO:38].

#### Example 5

##### Baculovirus expression plasmid for 1-153 c-mpl ligand/mouse Fc

The 1-153 c-mpl ligand PCR product (PCR#4) was digested with NcoI and EcoRI restriction enzymes (ca. 460 bp) for transfer to a Baculovirus expression vector. The expression vector, pMON26440, was digested with NcoI and EcoRI (ca. 10 Kb). pMON26440 is a derivative of pVL1393 (Invitrogen) containing a DNA sequence encoding a modified human IL-3 secretion signal peptide sequence (Example 3, above) and a DNA gene fragment encoding a modified mouse Fc (IgG2a) constant region and hinge region (Gross A.H., *J.Clin.Invest.* 95:2783-2789, [1995]). The DNA sequence, encoding the hIL-3 secretion signal peptide was subcloned into the BamHI site of the vector leaving the NcoI site at the 3' end of the signal sequence for clon-

ing. The mouse Fc gene fragment (EcoRI-BglIII) was subcloned into the BglIII site of the vector such that the EcoRI site was available for cloning at the 5' end of the Fc. This facilitates cloning genes as NcoI-EcoRI fragments into the vector. A termination codon is introduced in-frame between the gene of interest and the EcoRI site. The EcoRI site (GAATTC) is added directly 3' to the gene of interest such that it encodes glutamate (GAA) and phenylalanine (TTC) codons. Following the EcoRI site is a DNA sequence encoding a Factor Xa proteolytic cleavage site, a small polypeptide linker region which is followed by the mouse Fc gene fragment. Plasmid DNA was prepared from individual clones and the c-mpl ligand insert portion were sequenced. pMON26454-4, encoding a chimera protein consisting of 1-153  $\Delta$ 112-115 c-mpl ligand joined to a mouse Fc, is a result of this cloning event. The plasmid, pMON26454-4, contains the DNA sequence [SEQ ID NO:50] which encodes the polypeptide represented by [SEQ ID NO:41]. pMON26454-8, encoding a chimera protein consisting of amino acids 1-153 c-mpl ligand joined to a mouse Fc, is also a result of this cloning event. The plasmid, pMON26454-8, contains the DNA sequence [SEQ ID NO:61] which encodes the polypeptide represented by [SEQ ID NO:68].

#### Example 6

##### BHK expression plasmid for 1-153 c-mpl ligand $\Delta$ 112-115/mouse Fc

In order to create a BHK plasmid for expression of 1-153 c-mpl ligand  $\Delta$ 112-115/mouse Fc, the NcoI-PstI fragment (ca. 310 bp) of pMON26448 was combined with the PstI-EcoRI fragment (ca. 150 bp) of pMON26454-4 for ligation to the NcoI-EcoRI vector fragment of pMON3993 (ca. 4550 bp.). pMON3993 is a derivative of pMON3934 (above) and contains a modified mouse Fc gene fragment. An EcoRI-

BglII mouse Fc gene fragment was transferred into the vector 3' to the NcoI site, leaving an EcoRI site between the NcoI site and the mouse Fc gene. An EcoRI site elsewhere in the vector had previously been destroyed. This facilitates cloning genes as NcoI-EcoRI fragments into the vector and fuses the gene of interest in-frame to the mouse Fc. A termination codon was introduced in-frame between the gene of interest and the EcoRI site. The EcoRI site (GAATTC) is added directly 3' to the gene of interest such that it encodes glutamate (GAA) and phenylalanine (TTC) codons. pMON26465, encoding amino acids 1-153  $\Delta$ 112-115 c-mpl ligand joined to a mouse Fc, is a result of this cloning.

The plasmid, pMON26465, contains the DNA sequence [SEQ ID NO:51] which encodes the polypeptide represented by [SEQ ID NO:42].

#### Example 7

BHK expression plasmid for 1-153 c-mpl ligand  $\Delta$ 112-115

A second BHK expression plasmid encoding amino acids 1-153  $\Delta$ 112-115 c-mpl ligand was constructed in which the DNA sequence at the 5' end of the 1-153  $\Delta$ 112-115 c-mpl ligand gene was changed, without altering the resulting amino acid sequence, to optimize protein expression which might result in increased secretion of the protein. This plasmid, pMON30214, was constructed by ligating the NcoI-BamHI fragment of pMON26465 (ca. 370 bp), the BamHI-HindIII fragment of pMON26448 (ca. 90 bp) and the NcoI-HindIII fragment of pMON3934 (ca. 3800 bp). Plasmid DNA was prepared from individual clones and the c-mpl ligand insert portion were sequenced. The plasmid, pMON30214, contains the DNA sequence [SEQ ID NO:48] which encodes the polypeptide represented by [SEQ ID NO:38].

#### Example 8

E. coli expression plasmid for 1-153 c-mpl ligand/glyser/IL-3 variant 13288

5 pMON26461, encoding the chimera protein, 1-153 c-mpl  
ligand/glyser/IL-3 variant 13288 (WO 94/12638), was  
constructed by ligating the NcoI-EcoRI fragment of  
pMON26454-8 (ca. 460 bp.) and a synthetic linker (EcoSnaI  
[SEQ ID NO:30], EcoSna2 [SEQ ID NO:31]) to the SnaBI-NcoI  
10 vector fragment (ca. 3500 bp.) of pMON13057 [WO 9  
5/21254]. The plasmid, pMON26461, contains the DNA se-  
quence [SEQ ID NO:72] which encodes the polypeptide  
represented by [SEQ ID NO:73].

15

Example 9

BHK expression vector for 1-153 c-mpl ligand/FXa/glyser/IL-3 variant 13288

20 pMON26474, encoding the chimera protein, 1-153 c-mpl  
ligand/FXa/glyser/IL-3 variant 13288, was constructed by  
ligating the NcoI-HindIII fragment of pMON26472 (ca. 860  
bp.) to the NcoI-HindIII fragment of pMON3934 (ca. 3800).  
pMON26472, an E. coli expression plasmid for amino acids  
1-153 c-mpl ligand/FXa/glyser/IL-3 variant 13288, was  
25 constructed by combining the NcoI-SnaBI fragment of  
pMON26461 (ca. 460 bp.) and the SnaBI-HindIII fragment of  
pMON3988 (ca. 400 bp.) (WO 95/21254) for ligation to the  
NcoI-HindIII vector fragment of pMON3935 (ca. 3250 bp.).  
The plasmid, pMON26474, contains the DNA sequence [SEQ ID  
30 NO:64] which encodes the polypeptide represented by [SEQ  
ID NO:71].

35

Example 10

BHK expression plasmid for 1-153 A112-115 c-mpl ligand/FXa/glyser/IL-3 variant 13288

pMON26469, encoding the chimera protein, 1-153  $\Delta$ #112-115 c-mpl ligand joined to IL-3 variant 13288, was constructed. The NcoI-BamHI fragment from pMON26454-4 (ca. 370 bp) was combined with the BamHI-HindIII fragment of pMON26474 (ca. 490 bp) and the NcoI-HindIII vector fragment of pMON3934 (ca. 3800 bp). The plasmid, pMON26469, contains the DNA sequence [SEQ ID NO:52] which encodes the polypeptide represented by [SEQ ID NO:43].

#### Example 11

BHK expression plasmid for 1-153  $\Delta$ 112-115 c-mpl lig and/IL-3 variant 13288 chimera

pMON30243, encoding the chimera protein, 1-153  $\Delta$ 112-115 c-mpl ligand joined to IL-3 variant 13288, was constructed by combining the NcoI-SnaBI fragment of pMON26469 (ca. 460 bp) with the SnaBI-HindIII fragment (ca. 400 bp) of pMON26427 (WO 95/21254) and ligating to the NcoI-HindIII fragment (ca. 3800 bp) of pMON3934. The plasmid, pMON30243, contains the DNA sequence [SEQ ID NO:53] which encodes the polypeptide represented by [SEQ ID NO:44].

#### Example 12

E. coli expression plasmid for IL-3 variant 13288 /FXa/glyser/1-153 c-mpl ligand

pMON26460, an E. coli expression plasmid for production of the chimera protein, IL-3 variant 13288 /FXa/glyser/amino acids 1-153 c-mpl ligand, was constructed by ligating the NcoI-HindIII fragment of pMON26448 (ca. 460 bp.) to the AflIII-HindIII vector fragment (ca. 3500 bp) of pMON13018 (WO 95/21254). The plasmid, pMON26460, contains the DNA sequence [SEQ ID NO:62] which encodes the polypeptide represented by [SEQ ID NO:69].

Example 13E. coli expression plasmid for IL-3 variant 13288  
/glyser/1-153 c-mpl ligand

5 pMON26471, an *E. coli* expression plasmid for the produc-  
tion of the chimera protein, IL-3 variant 13288  
/glyser/1-153 c-mpl ligand, was constructed by ligating  
the NcoI-SmaI fragment of pMON26426 (ca. 370 bp.) to the  
SmaI-HindIII fragment of pMON26460 (ca. 490 bp) into the  
10 NcoI-HindIII vector fragment of pMON3935 (3250 bp.).  
pMON26426 was constructed by transferring the NcoI-  
HindIII fragment (ca. 950 bp.) of pMON13056 (WO 95/21254)  
to the NcoI-HindIII vector fragment (ca. 3800 bp.) of  
pMON3934 (ca. 3800 bp.). The plasmid, pMON26471, contains  
15 the DNA sequence [SEQ ID NO:63] which encodes the poly-  
peptide represented by [SEQ ID NO:70].

Example 14

20

BHK expression plasmid for IL-3 variant 13288 /glyser/1-  
153 A112-115 c-mpl ligand

pMON30272, expressing IL-3 variant 13288 joined via a  
glycine-serine linker to amino acids 1-153 A112-115 c-mpl  
25 ligand, was constructed by combining the NcoI-PstI frag-  
ment of pMON26473 (ca. 700 bp) with the PstI-HindIII  
fragment of pMON30214 (ca. 160 bp) and ligating to the  
NcoI-HindIII fragment of pMON3934 (ca. 3800 bp).  
pMON26473 was constructed by ligating the NcoI-HindIII  
30 fragment of pMON26471 (ca. 880 bp.) to the NcoI-HindIII  
vector fragment of pMON3934 (ca. 3800 bp.). The plasmid,  
pMON30272, contains the DNA sequence [SEQ ID NO:54] which  
encodes the polypeptide represented by [SEQ ID NO:45].

35

Example 15



BHK expression plasmid for 1-153  $\Delta$ 112-115/his<sub>6</sub> c-mpl  
ligand

pMON30253, expressing 1-153  $\Delta$ 112-115/his<sub>6</sub> c-mpl ligand,  
was constructed by digesting pMON26465 with EcoRI and  
ligation of a synthetic linker that adds a C-terminal  
poly-histidine tag. The linker was constructed by an-  
nealing the HisC1 [SEQ ID NO:32] and HisC2 [SEQ ID NO:33]  
primers which creates a GluPheHisHisHisHisHisHis [SEQ ID  
NO:57] tail immediately following the c-mpl ligand mole-  
cule. A termination codon following the last His codon  
prevents translation beyond that point. The DNA was  
sequenced to confirm orientation of the linker which  
keeps a unique EcoRI site immediately 3' to the c-mpl  
ligand gene and 5' to the poly-histidine tail. The plas-  
mid, pMON30253, contains the DNA sequence [SEQ ID NO:55]  
which encodes the polypeptide represented by [SEQ ID  
NO:46].

Example 16

BHK expression plasmid for his<sub>6</sub>/1-153  $\Delta$ 112-115 c-mpl  
ligand

pMON30274, expressing his<sub>6</sub>/1-153  $\Delta$ 112-115 c-mpl ligand,  
was constructed by digesting pMON30214 with NcoI and  
ligation of a synthetic linker that adds an N-terminal  
poly-histidine tag. The linker was constructed by an-  
nealing the HisN1 [SEQ ID NO:34] and HisN2 [SEQ ID NO:35]  
primers which creates a HisHisHisHisHisHisAlaMetAla [SEQ  
ID NO:36] tag immediately proceeding the c-mpl ligand  
molecule. The DNA was sequenced to confirm the orienta-  
tion of the linker which keeps a unique NcoI site 3' to  
the tag and 5' to the c-mpl ligand gene. The plasmid,  
pMON30274, contains the DNA sequence [SEQ ID NO:56] which  
encodes the polypeptide represented by [SEQ ID NO:47].

Example 17Assembling genes from previous examples

5 A variety of different of c-mpl ligand genes can be assembled from Examples 1 through 6 by combining various gene fragments via restriction enzyme digestion and ligation (see Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, [1989]). The genes from  
10 the examples can be digested with restriction enzymes on either end of the gene as well as internally to release desired gene fragments. Then, the different pieces can be ligated together and to an expression vector through complementary ends using DNA ligase. The ligated DNA can  
15 be transformed into *E. coli* and colonies are screened for the desired gene product through DNA sequencing of plasmid DNA. After identification of positive clones, the plasmid DNA can be transfected into an appropriate mammalian cell or *E. coli* strain for production.

20

Example 18Site-directed mutagenesis

25 A variety of amino acid substitutions can be made at each position using either synthetic gene assembly or site-directed mutagenesis (see Taylor et al., *Nucl. Acids Res.*, 13: 7864-8785 [1985]; Kunkel et al., *Proc. Natl. Acad. Sci. USA*, 82: 488-492 [1985]; Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold  
30 Spring Harbor Laboratory Press, Cold Spring Harbor, NY, [1989], (WO 94/12639) and (WO 94/12638)). These substitutions can be made one at a time or in combination with other amino acid substitutions. After sequence verification of the changes, the plasmid DNA can be transfected  
35 into an appropriate mammalian cell or *E. coli* strain for production.

Example 19SDS Folding Protocol For c-mpl Chimera Proteins

## 5      A. Recovery of c-mpl chimera protein from inclusion bodies.

10      *E. coli* cells from a 300ml culture expressing the chimera protein are resuspended in 150 mls of 20 mM Tris-HCl, 5mM EDTA, pH 8.0. The cell resuspension is sonicated by standard means for ~3 minutes on ice, and then centrifuged at 5,000 xg for 30 minutes. The recovered inclusion body (IB) pellet is washed 2X by resuspending in 150 mls of 20 mM Tris-HCl, 1 mM EDTA, pH 8.0, sonicating as  
15      above, and centrifuging at 5000 xg for 30 minutes. The washed IB pellet can be used immediately or stored at -70°C. A Manton-Gaulin homogenizer can also be used to disrupt larger quantities of cells when processing IB material in a similar manner at a larger scale.

20

## B. Solubilization and refold of monomeric c-mpl ligand Chimera Protein.

25      The washed IB pellet is resuspended in 100 mM Tris-HCl, pH 9.0 (pH 8.0-9.75 suitable) containing 0.1% (w/v) sodium dodecyl sulfate (SDS) at a ratio of 50 mls per ~1 gm of cell pellet using a hand tissue homogenizer. Dilute suspension to 200 mls in same buffer and stir at 4°C until completely mixed (5-15 minutes). Dithiothreitol  
30      (DTT) is added to a final conc. of 20 mM and L-cysteine (Sigma #4820) to a final conc. of 1 mM (DTT added from a fresh 50x stock, and cysteine added from a fresh 100x stock; stocks prepared in 100 mM Tris-HCl pH 9.00). The refold solution is air oxidized at 4°C (4°C to 25°C  
35      suitable) with moderate stirring in a loosely covered container (18-72 hrs). Refold is monitored by rHPLC analysis to determine completion.

Example 20Determination of bioactivity of c-mpl ligands and chimera molecules

pMON26448 (1-332 c-mpl ligand) and hIL-3 variant, pMON13288 (WO 94/12638), are used as activity reference standards in the following assays.

1. TF1 proliferation assay

c-mpl ligand proliferative activity is assayed using a subclone of the pluripotential human cell line TF1 (Kitamura et al., *J. Cell Physiol* 140:323-334. [1989]). TF1 cells are maintained in h-IL3 (100 U/mL). To establish a sub-clone responsive to c-mpl ligand, cells are maintained in passage media containing 10% supernatant from BHK cells transfected with the gene expressing the 1-153 form of c-mpl ligand (pMON26448). Most of the cells die, but a subset of cells survive. After dilution cloning, a c-mpl ligand responsive clone is selected, and these cells are split into passage media to a density of  $0.3 \times 10^6$  cells/mL the day prior to assay set-up. Passage media for these cells is the following: RPMI 1640 (Gibco), 10% FBS (Harlan, Lot #91206), 10% c-mpl ligand supernatant from transfected BHK cells, 1 mM sodium pyruvate (Gibco), 2 mM glutamine (Gibco), and 100 ug/mL penicillin-streptomycin (Gibco). The next day, cells are harvested and washed twice in RPMI or IMDM media with a final wash in the ATL, or assay media. ATL media consists of the following: IMDM (Gibco), 500 ug/mL of bovine serum albumin, 100 ug/mL of human transferrin, 50 ug/mL soybean lipids,  $4 \times 10^{-8}$ M beta-mercaptoethanol and 2 mL of A9909 (Sigma, antibiotic solution) per 1000 mL of ATL. Cells are diluted in assay media to a final density of  $0.25 \times 10^6$  cells/mL in a 96-well low evaporation plate (Costar) to a final volume of 50 ul. Transient

supernatants (conditioned media) from transfected clones are added at a volume of 50 ul as duplicate samples at a final concentration of 50% and diluted three-fold to a final dilution of 1.8%. Triplicate samples of a dose curve of IL-3 variant pMON13288 starting at 1 ng/mL and diluted using three-fold dilutions to 0.0014ng/mL is included as a positive control. Plates are incubated at 5% CO<sub>2</sub> and 37° C. At day six of culture, the plate is pulsed with 0.5 Ci of <sup>3</sup>H/well (NEN) in a volume of 20 ul/well and allowed to incubate at 5% CO<sub>2</sub> and 37° C for four hours. The plate is harvested and counted on a Betaplate counter.

Table 1  
TF1 Proliferation Assay

transfection pMON #	c-mpl ligand	TOTAL COUNTS tritiated thymidine incorporation 10% supernatants	Standard Deviation
mock		21910	561
pMON26448	1-153	114888	5269
pMON26450	1-153 Δ112-115	60827	1741
pMON26451-1	1-332 Glu <sup>122</sup>	133722	10040
pMON26451-3	1-332 Δ112-115	35600	3627
pMON26451-4	1-332 Leu <sup>46</sup> , Arg <sup>200</sup>	121684	2611

Table 1. The c-mpl ligands listed above were tested in the TF1 proliferation assay. All of the c-mpl ligands tested were active in this assay.

Other in vitro cell based assays may also be useful to determine the activity of the c-mpl ligands or chimera molecules depending on the colony stimulating factors that comprise the chimera. The following are examples of other useful assays. In addition blocking monoclonal antibodies, raised against one of the components of a chimera, can be used in a cell proliferation assay to determine that each component of a chimera is active.

32D proliferation assay: 32D is a murine IL-3 dependent cell line which does not respond to human IL-3 but does respond to human G-CSF which is not species restricted.

Baf/3 proliferation assay: Baf/3 is a murine IL-3 dependent cell line which does not respond to human IL-3 or human c-mpl ligand but does respond to human G-CSF which is not species restricted.

T1165 proliferation assay: T1165 cells are a IL-6 dependent murine cell line (Nordan et al., 1986) which respond to IL-6 and IL-11.

AML193 proliferation assay: This cell line, established from a patient with acute myelogenous leukemia, is a growth factor dependent cell line which displayed enhanced growth in GM-CSF supplemented medium (Lange, B., et al., (1987); Valtieri, M., et al., (1987)). The AML cell line also responds to human IL-3 and G-CSF.

Transfected cell lines: Cell lines such as Baf/3 cell line can be transfected with a colony stimulating factor receptor, such as the human IL-3 receptor or human c-mpl receptor, which the cell line does not have. These transfected cell lines can be used to determine the activity of the ligand for which the receptor has been transfected into the cell line.

One such transfected Baf/3 cell line was made by cloning the cDNA encoding c-mpl from a library made from a c-mpl responsive cell line and cloned into the multiple cloning site of the plasmid pcDNA3 (Invitrogen, San Diego Ca.). Baf/3 cells were transfected with the plasmid via electroporation. The cells were grown under G418 selection in the presence of mouse IL-3 in Wehi conditioned media. Clones were established through limited dilution.

Table 2  
AML and TF1 Proliferation Assay  
of c-mpl ligand/Fc chimera

factor	Baf/3-cmpl-R Cells tested at 1 $\mu$ g/ml	TF1.2.B4 Cells tested at 2 $\mu$ g/ml
pMON26465	10470 +/- 700	4400 +/- 110
pMON26458	25000 +/- 580	20300 +/- 1200
CTLA-4 mFc control	1020 +/- 120	580 +/- 260

Table 2. pMON26465 and pMON26458 were assayed for proliferative activity in both Baf/3 cells transfected with the human c-mpl-R (Baf/3-cmpl-R) and in the TF1.2.B4 cell line. Cells were pulsed with 1 $\mu$ Ci/well of tritiated thymidine for overnight (Baf/3-cmpl-R cells) or for 4 hours (TF1.2.B4 cells) and total counts cpm (3H) determined on a beta plate counter.

## 2. Bone marrow proliferation assay

### a. CD34+ Cell Purification:

Between 15-20 mL bone marrow aspirates are obtained from normal allogeneic marrow donors after informed consent. Cells are diluted 1:3 in phosphate buffered saline (PBS, Gibco-BRL), 30 mL are layered over 15 mL Histopaque-1077 (Sigma) and centrifuged for 30 minutes at 300 RCF. The mononuclear interface layer is collected and washed in PBS. CD34+ cells are enriched from the mononuclear cell preparation using an affinity column per manufacturers instructions (CellPro, Inc, Bothell WA). After enrichment, the purity of CD34+ cells is 70% on average as determined by using flow cytometric analysis using anti-CD34 monoclonal antibody conjugated to fluorescein and anti-CD38 conjugated to phycoerythrin (Becton Dickinson, San Jose CA).

Cells are resuspended at 40,000 cells/mL in X-Vivo 10 media (Bio-Whittaker, Walkersville, MD) and 1 mL is plated in 12-well tissue culture plates (Costar). Human IL-3 variant, pMON13288, is used at 10 ng/mL or 100 ng/mL. Conditioned media from BHK cells transfected with plasmid encoding c-mpl ligand are tested by addition of 100 µl of supernatant added to 1 mL cultures (approximately a 10% dilution). Cells are incubated at 37°C for 8-14 days at 5% CO<sub>2</sub> in a 37°C humidified incubator.

#### b. Cell Harvest and Analysis:

At the end of the culture period a total cell count is obtained for each condition. For fluorescence analysis and ploidy determination cells, are washed in megakaryocyte buffer (MK buffer, 13.6 mM Sodium Citrate, 1 mM Theophylline, 2.2 µM PGE<sub>1</sub>, 11 mM Glucose, 3% w/v BSA, in PBS, pH 7.4,) (Tomer et al., *Blood* 70(6): 1735-42 [1987]) resuspended in 500 µl of MK buffer containing anti-CD41a FITC antibody (1:200, AMAC, Westbrook, ME) and washed in MK buffer. For DNA analysis cells are made permeable in MK buffer containing 0.5% Tween 20 (Fisher, Fair Lawn NJ) for 20 minutes on ice followed by fixation in 0.5% Tween-20 and 1% paraformaldehyde (Fisher Chemical) for 30



minutes followed by incubation in Propidium Iodide (Calbiochem , La Jolla Ca) (50 µg/mL) with RNAase (400 U/mL) in 55% v/v MK buffer (200 mOsm) for 1-2 hours on ice. Cells are analyzed on a FACScan or Vantage flow cytometer (Becton Dickinson, San Jose, CA). Green fluorescence (CD41a-FITC) is collected along with linear and log signals for red fluorescence (PI) to determine DNA ploidy. All cells are collected to determine the percent of cells that are CD41+. Data analysis is performed using LYSIS software (Becton Dickinson, San Jose, CA). Percent of cells expressing the CD41 antigen is obtained from flow cytometry analysis (Percent). Absolute (Abs) number of CD41+ cells/mL is calculated by:  $(\text{Abs}) = (\text{Cell Count}) * (\text{Percent}) / 100$ .

Table 3  
Bone Marrow Proliferation Assay

Proliferation of CD34+ enriched cells in the presence of c-mpl ligand variants		
Transfection Construct	c-mpl ligand	Cells/Well after 10 Day Culture (4000 cells plated)
Mock Control		<500
pMON26448	1-153	22,500
pMON26450	1-153 $\Delta$ 112-115	<500
pMON26451-3	1-332 Glu <sup>122</sup>	<500
pMON26451-4	1-332 leu <sup>46</sup> , Arg <sup>200</sup>	16,000

5

Table 3. The c-mpl ligands listed above were tested in the megakaryocyte liquid culture assay. pMON26448 and pMON26451-4 were active in this assay.

Table 4  
Megakaryocyte liquid culture assay

Absolute number of CD41+ cells after 10 days in culture in the presence or absence of IL-3 pMON13288			
Transfection Construct	c-mpl ligand	No pMON13288	pMON13288 (10 ng/mL)
Mock Control		<500	3000
pMON26448	1-153	19,000	45,000
pMON26450	1-153 $\Delta$ 112-115	<500	8,500
pMON26451-3	1-332 Glu <sup>122</sup>	<500	9,000
pMON26451-4	1-332 leu <sup>46</sup> , Arg <sup>200</sup>	12,500	16,000

Table 4. The c-mpl ligands listed above were tested in the megakaryocyte liquid culture assay as described above. pMON26450 and pMON26451-3 were not active by themselves but were active when co-administered with the human IL3 receptor agonist, pMON13288, over and above pMON13288 by itself.

### 3. Megakaryocyte fibrin clot assay.

CD34+ enriched population are isolated as described above. Cells are suspended at 25,000 cells/mL with/without cytokine(s) in a media consisting of a base Iscoves IMDM media supplemented with 0.3% BSA, 0.4 mg/mL apo-transferrin, 6.67  $\mu$ M FeCl<sub>2</sub>, 25  $\mu$ g/mL CaCl<sub>2</sub>, 25  $\mu$ g/mL L-asparagine, 500  $\mu$ g/mL E-amino-n-caproic acid and Penicillin/Streptomycin. Prior to plating into 35 mm plates, thrombin is added (0.25 units/mL) to initiate clot formation. Cells are incubated at 37°C for 13 days at 5% CO<sub>2</sub> in a 37°C humidified incubator. At the end of the culture period plates are fixed with methanol:acetone (1:3), air dried and stored at -200C until staining. A peroxidase immunocytochemistry staining procedure is used

(Zymed, Histostain-SP. San Francisco, CA) using a cocktail of primary monoclonal antibodies consisting of anti-CD41a, CD42 and CD61. Colonies are counted after staining and classified as negative, CFU-MK (small colonies, 1-2 foci and less than approx. 25 cells), BFU-MK (large, multi-foci colonies with > 25 cells) or mixed colonies (mixture of both positive and negative cells).

5

10

## WHAT IS CLAIMED IS:

- 5                   1.           A c-mpl ligand of the following formula:

SerProAlaProProAlaCysAspLeuArgValLeuSerLysLeuLeuArgAspSer  
1                   5                   10                   15

10           HisValLeuHisSerArgLeuSerGlnCysProGluValHisProLeuProXaaPro  
20                   25                   30                   35

ValLeuLeuProAlaValAspXaaXaaLeuGlyGluTrpLysThrGlnMetGluGlu  
40                   45 46                   50                   55

15           ThrLysAlaGlnAspIleLeuGlyAlaValThrLeuLeuLeuGluGlyValMetAla  
60                   65                   70                   75

20           AlaArgGlyGlnLeuGlyProThrCysLeuSerSerLeuLeuGlyGlnLeuSerGly  
80                   85                   90                   95

GlnValArgLeuLeuLeuGlyAlaLeuGlnSerLeuLeuGlyThrGlnXaaXaaXaa  
100                   105                   110

25           XaaGlyArgThrThrAlaHisXaaAspProAsnAlaIlePheLeuSerPheGlnHis  
115                   120   122                   125                   130

LeuLeuArgGlyLysValArgPheLeuMetLeuValGlyGlySerThrLeuCysVal  
135                   140                   145                   150

30           ArgArgAlaProProThrThrAlaValProSerArgThrSerLeuValLeuThrLeu  
155                   160                   165                   170

35           AsnGluLeuProAsnArgThrSerGlyLeuLeuGluThrAsnPheThrAlaSerAla  
175                   180                   185                   190

ArgThrThrGlySerGlyLeuLeuLysXaaGlnGlnGlyPheArgAlaLysIlePro  
195                   200                   205

GlyLeuLeuAsnGlnThrSerArgSerLeuAspGlnIleProGlyTyrLeuAsnArg  
 210                      215                      220                      225

5      IleHisGluLeuLeuAsnGlyThrArgGlyLeuPheProGlyProSerArgArgThr  
          230                      235                      240                      245

LeuGlyAlaProAspIleSerSerGlyThrSerAspThrGlySerLeuProProAsn  
          250                      255                      260                      265

10     LeuGlnProGlyTyrSerProSerProThrHisProProThrGlyGlnTyrThrLeu  
          270                      275                      280                      285

PheProLeuProProThrLeuProThrProValValGlnLeuHisProLeuLeuPro  
 15                      290                      295                      300

AspProSerAlaProThrProThrProThrSerProLeuLeuAsnThrSerTyrThr  
 305                      310                      315                      320

20     HisSerGlnAsnLeuSerGlnGluGly  
          325                      330      332

wherein;

- 25     Xaa at position 37 is Thr, Asp or Glu;  
        Xaa at position 46 is Phe, Ala, Val, Leu, Ile, Pro, Trp,  
        or Met;  
        Xaa at position 47 is Ser, Asp or Glu;  
        Xaa at position 112 is deleted or Leu, Ala, Val, Ile,  
 30     Pro, Phe, Trp, or Met;  
        Xaa at position 113 is deleted or Pro, Phe, Ala, Val,  
        Leu, Ile, Trp, or Met;  
        Xaa at position 114 is deleted or Pro, Phe, Ala, Val,  
        Leu, Ile, Trp, or Met;  
 35     Xaa at position 115 is deleted or Gln, Gly, Ser, Thr,  
        Tyr, or Asn;

Xaa at position 122 is Lys, Arg, His, Glu, or Asp;  
Xaa at position 200 is Trp, Ala, Val, Leu, Ile, Pro, Phe,  
Met, Arg, Lys, or His;

5 wherein from 1 to 179 amino acids can be deleted from the  
C-terminus and with the proviso that at least one of the  
amino acids designated by Xaa are different from the  
corresponding amino acids of native c-mpl ligand (1-332)  
and said protein is optionally preceded by Met<sup>-1</sup>, Ala<sup>-1</sup> or  
10 Met<sup>-2</sup> Ala<sup>-1</sup>.

2. The c-mpl ligand of Claim 1 wherein;

Xaa at position 37 is Thr, Asp or Glu;  
15 Xaa at position 46 is Phe, Ala, Val, Leu, Ile, Pro, Trp,  
or Met;  
Xaa at position 47 is Ser, Asp or Glu;  
Xaa at position 112 is deleted;  
Xaa at position 113 is deleted;  
20 Xaa at position 114 is deleted;  
Xaa at position 115 is deleted;  
Xaa at position 122 is Lys, Arg, His, Glu, or Asp;  
Xaa at position 200 is Trp, Ala, Val, Leu, Ile, Pro, Phe,  
Met, Arg, Lys, or His;

25 wherein amino acids 112-115 are deleted and from 1 to 179  
amino acids can be deleted from the C-terminus and  
optionally that one or more of the amino acids designated  
by Xaa are different from the corresponding amino acids  
30 of native c-mpl ligand (1-332) and which is optionally  
preceded by Met<sup>-1</sup>, Ala<sup>-1</sup> or Met<sup>-2</sup> Ala<sup>-1</sup>.

3. The c-mpl ligand of Claim 1 wherein;

35 Xaa at position 37 is Thr, Asp or Glu;

Xaa at position 46 is Ala, Val, Leu or Ile;  
Xaa at position 47 is Ser, Asp or Glu;  
Xaa at position 112 is deleted or Ala, Val, Ile or Phe;  
Xaa at position 113 is deleted or Phe, Ala, Val, Leu,  
5 Ile, Trp, or Met;  
Xaa at position 114 is deleted or Phe, Ala, Val, Leu,  
Ile, Trp, or Met;  
Xaa at position 115 is deleted or Asn;  
Xaa at position 122 is Arg, His, Glu, or Asp;  
10 Xaa at position 200 is Ala, Val, Leu, Ile, Phe or Met.

4. The c-mpl ligand of Claim 1 wherein;

Xaa at position 37 is Thr, Asp or Glu;  
15 Xaa at position 46 is Ala, Val, Leu or Ile;  
Xaa at position 47 is Ser, Asp or Glu;  
Xaa at position 112 is Ala, Val, Ile or Phe;  
Xaa at position 113 is Phe, Ala, Val, Leu, Ile, Trp, or  
Met;  
20 Xaa at position 114 is Phe, Ala, Val, Leu, Ile, Trp, or  
Met;  
Xaa at position 115 is Asn;  
Xaa at position 122 is Arg, His, Glu, or Asp;  
Xaa at position 200 is Ala, Val, Leu, Ile, Phe or Met.

25

5. The c-mpl ligand of Claim 1 wherein;

Xaa at position 37 is Thr, Asp or Glu;  
Xaa at position 46 is Leu or Phe;  
30 Xaa at position 47 is Ser, Asp or Glu;  
Xaa at position 112 is deleted or Leu;  
Xaa at position 113 is deleted or Pro;  
Xaa at position 114 is deleted or Pro;  
Xaa at position 115 is deleted or Gln;  
35 Xaa at position 122 is Lys or Glu;  
Xaa at position 200 is Trp or Arg.



6. The c-mpl ligand of Claim 1 wherein;  
 said Xaa at position 46 is Leu;  
 Xaa at position 112 is Leu;  
 5 Xaa at position 113 is Pro;  
 Xaa at position 114 is Pro;  
 Xaa at position 115 is Gln;  
 said Xaa at position 122 is Glu; and  
 said Xaa at position 200 is Arg.

7. The c-mpl ligand of Claim 1 having the  
 sequence of native c-mpl (1-332) with one of the  
 following mutation(s);  
 Glu at 122; or  
 15 Leu at 46 and Arg at 200; or  
 Arg at 200.

8. A c-mpl ligand of the following formula:

20 SerProAlaProProAlaCysAspLeuArgValLeuSerLysLeuLeuArgAspSer  
 1 5 10 15

HisValLeuHisSerArgLeuSerGlnCysProGluValHisProLeuProXaaPro  
 20 25 30 35

25 ValLeuLeuProAlaValAspXaaXaaLeuGlyGluTrpLysThrGlnMetGluGlu  
 40 45 46 50 55

ThrLysAlaGlnAspIleLeuGlyAlaValThrLeuLeuLeuGluGlyValMetAla  
 30 60 65 70 75

AlaArgGlyGlnLeuGlyProThrCysLeuSerSerLeuLeuGlyGlnLeuSerGly  
 80 85 90 95

35 GlnValArgLeuLeuLeuGlyAlaLeuGlnSerLeuLeuGlyThrGlnXaaXaaXaa  
 100 105 110

XaaGlyArgThrThrAlaHisXaaAspProAsnAlaIlePheLeuSerPheGlnHis  
 115                      120    122            125                      130

LeuLeuArgGlyLysValArgPheLeuMetLeuValGlyGlySerThrLeuCysVal  
 5                      135                      140                      145                      150

Arg  
 153

10            wherein;

Xaa at position 37 is Thr, Asp or Glu;  
 Xaa at position 46 is Phe, Ala, Val, Leu, Ile, Pro, Trp,  
 or Met;

15            Xaa at position 47 is Ser, Asp or Glu;  
 Xaa at position 112 is deleted or Leu, Ala, Val, Ile,  
 Pro, Phe, Trp, or Met;

Xaa at position 113 is deleted or Pro, Phe, Ala, Val,  
 Leu, Ile, Trp, or Met;

20            Xaa at position 114 is deleted or Pro, Phe, Ala, Val,  
 Leu, Ile, Trp, or Met;

Xaa at position 115 is deleted or Gln, Gly, Ser, Thr,  
 Tyr, or Asn;

Xaa at position 122 is Lys, Arg, His, Glu, or Asp;

25            with the proviso that at least one of the amino acids  
 designated by Xaa are different from the corresponding  
 amino acids of native c-mpl ligand (1-153) and which is  
 optionally preceded by Met<sup>-1</sup>, Ala<sup>-1</sup> or Met<sup>-2</sup> Ala<sup>-1</sup>.

30

9. The c-mpl ligand of Claim 8 wherein;

Xaa at position 37 is Thr, Asp or Glu;

35            Xaa at position 46 is Phe, Ala, Val, Leu, Ile, Pro, Trp,  
 or Met;

Xaa at position 47 is Ser, Asp or Glu;

Xaa at position 112 is deleted;

Xaa at position 113 is deleted;  
Xaa at position 114 is deleted;  
Xaa at position 115 is deleted;  
Xaa at position 122 is Lys, Arg, His, Glu, or Asp;

5

wherein one or more of the amino acids designated by Xaa  
are different from the corresponding amino acids of  
native c-mpl ligand (1-153) and said protein is  
optionally preceded by Met<sup>-1</sup>, Ala<sup>-1</sup> or Met<sup>-2</sup> Ala<sup>-1</sup>.

10

10. The c-mpl ligand of Claim 8 wherein;

15

Xaa at position 37 is Thr, Asp or Glu;  
Xaa at position 46 is Leu or Phe;  
Xaa at position 47 is Ser, Asp or Glu;  
Xaa at position 112 is deleted or Leu;  
Xaa at position 113 is deleted or Pro;  
Xaa at position 114 is deleted or Pro;  
Xaa at position 115 is deleted or Gln;  
Xaa at position 122 is Lys or Glu; and

20

5

11. The c-mpl ligand of Claim 8 having the  
sequence of native c-mpl ligand (1-153) with one of the  
following mutation(s);

Glu at 122; or

Leu at 46 and Arg at 200; or

Arg at 200.

10

12. A c-mpl ligand according to claim 1, having  
the protein sequence of [SEQ ID NO:37].

15

13. A c-mpl ligand according to claim 8, having  
the protein sequence of [SEQ ID NO:38].

14. A c-mpl ligand according to claim 1, which is a chimera to mouse Fc constant region, having the protein sequence selected from the group consisting of [SEQ ID NO:41] and [SEQ ID NO:42].

15. A chimera protein having the formula selected from the group consisting of  $R_1$ -L- $R_2$ ,  $R_2$ -L- $R_1$ ,  $R_1$ -L- $R_1$ ;  
wherein;

$R_1$  is a c-mpl ligand of claim 1;

$R_2$  is a colony stimulating factor selected from the group consisting of GM-CSF, CSF-1, G-CSF, M-CSF, erythropoietin (EPO), IL-1, IL-2, IL-3, a hIL-3 variant, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-16, LIF, flt3/flk2, human growth hormone, B-cell growth factor, B-cell differentiation factor, eosinophil differentiation factor and stem cell factor (SCF); and

L is a linker capable of linking  $R_1$  and  $R_2$ .

16. A chimera protein having the formula selected from the group consisting of  $R_1$ -L- $R_2$ ,  $R_2$ -L- $R_1$ ,  $R_1$ -L- $R_1$ ;  
wherein;

$R_1$  is a c-mpl ligand of claim 8;

$R_2$  is a colony stimulating factor selected from the group consisting of GM-CSF, CSF-1, G-CSF, M-CSF, erythropoietin (EPO), IL-1, IL-2, IL-3, a hIL-3 variant, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-16, LIF, flt3/flk2, human growth

hormone, B-cell growth factor, B-cell differentiation factor, eosinophil differentiation factor and stem cell factor (SCF); and

5 L is a linker capable of linking  $R_1$  and  $R_2$ .

17. The chimera protein according to claim 16, having the amino acid sequence selected from the group consisting of [SEQ ID NO:43], [SEQ ID NO:44], and [SEQ ID  
10 NO:45].

18. A recombinant DNA sequence comprising vector DNA and a DNA that encodes a ligand of claim 8, having the DNA sequence selected from the group consisting of  
15 [SEQ ID NO:40], [SEQ ID NO:48], [SEQ ID NO:49].

19. A recombinant DNA sequence comprising vector DNA and a DNA that encodes a ligand of claim 12, having the DNA sequence of [SEQ ID NO:39].  
20

20. A recombinant DNA sequence comprising vector DNA and a DNA that encodes a ligand of claim 14, having the DNA sequence selected from the group consisting of [SEQ ID NO:50] and [SEQ ID NO:51]  
25

21. A recombinant DNA sequence comprising vector DNA and a DNA that encodes a ligand of claim 16, having the DNA sequence selected from the group consisting of [SEQ ID NO:52], [SEQ ID NO:53], [SEQ ID NO:54].  
30

22. A method of producing a variant c-mpl ligand comprising the steps of

(a) culturing a host cell containing a recombinant  
35 DNA sequence comprising vector DNA and a DNA sequence that encodes the c-mpl ligand having the amino acid sequence of a polypeptide selected from the group

consisting of a polypeptide of claim 1, a polypeptide of claim 2, a polypeptide of claim 3, a polypeptide of claim 4, a polypeptide of claim 5, a polypeptide of claim 6, a polypeptide of claim 7, a polypeptide of claim 8, a polypeptide of claim 9, a polypeptide of claim 10, a polypeptide of claim 11, a polypeptide of claim 12, a polypeptide of claim 13, a polypeptide of claim 14, a polypeptide of claim 15, a polypeptide of claim 16, polypeptide of claim 17 and capable under conditions permitting expression of the encoded polypeptide from the recombinant DNA; and

(b) harvesting the polypeptide from the culture.

23. A pharmaceutical composition comprising a therapeutically effective amount of a c-mpl ligand of claim 1, 2, 8, 9, 11, 12, 13, 14, 15, 16 or 17 together with a pharmaceutically acceptable carrier.

24. A pharmaceutical composition comprising;  
a therapeutically effective amount of a c-mpl ligand of claim 1;

a colony stimulating factor selected from the group consisting of GM-CSF, CSF-1, G-CSF, M-CSF, erythropoietin (EPO), IL-1, IL-2, IL-3, a hIL-3 variant, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-16, LIF, flt3/flk2, human growth hormone, B-cell growth factor, B-cell differentiation factor, eosinophil differentiation factor and stem cell factor (SCF); and

a pharmaceutically acceptable carrier.

25. A pharmaceutical composition comprising;  
a therapeutically effective amount of a c-mpl ligand of

claim 8;

a colony stimulating factor selected from the group consisting of GM-CSF, CSF-1, G-CSF, M-CSF, erythropoietin (EPO), IL-1, IL-2, IL-3, a hIL-3 variant, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, LIF, flt3/flk2, human growth hormone, B-cell growth factor, B-cell differentiation factor, eosinophil differentiation factor and stem cell factor (SCF); and

a pharmaceutically acceptable carrier.

26. A method of stimulating the production of hematopoietic cells which comprises administering a therapeutically effective amount of a c-mpl ligand of claim 1, 2, 8, 9, 11, 12, 13, 14, 15, 16 or 17 to a patient in need of such treatment.

27. A method for selective ex vivo expansion of stem cells, comprising the steps of; (a) separating stem cells from other cells; (b) culturing said separated stem cells with a selected media which comprises; a c-mpl ligand of claim 1, 2, 8, 9, 11, 12, 13, 14, 15, 16 or 17; (c) harvesting said cultured cells.

28. A method for treatment of a patient having a hematopoietic disorder, comprising the steps of; (a) removing stem cells; (b) separating stem cells from other cells; (c) culturing said separated stem cells with a selected media which comprises; a c-mpl ligand of claim 1, 2, 8, 9, 11, 12, 13, 14, 15, 16 or 17; (d) harvesting said cultured cells.

29. A method of human gene therapy comprising the steps of; (a) removing stem cells; (b) separating said stem cells from other cells; (c) introducing DNA

into said separated stem cells; (d) culturing said  
separated stem cells with a selected media comprising; a  
c-mpl ligand of claim 1, 2, 8, 9, 11, 12, 13, 14, 16, or  
17; (e) harvesting said cultured cells; and (f)  
5 transplanting said harvested cells into a patient in need  
of such treatment.

30. A method of stimulating the production of  
hematopoietic cells of a blood donor comprising the step  
10 of; (a) administering a therapeutically effective amount  
of a c-mpl ligand of claim 1, 2, 8, 9, 11, 12, 13, 14,  
15, 16 or 17; and (b) collection of hematopoietic cells  
from said blood donor.

15



# INTERNATIONAL SEARCH REPORT

International Application No.

PC1/US 96/00830

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/62 C07K14/52 C07K19/00 C07K14/54 A61K38/19  
A61K38/20 A61K48/00 C12N15/19 C12N15/24 C12N15/13

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 91, December 1994 WASHINGTON US, pages 13023-13027,	1,3,22
Y	D. FOSTER ET AL 'Human thrombopoietin: gene structure, cDNA sequence, expression, and chromosomal localization' see the whole document --- -/--	15,23-30

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search

11 April 1996

Date of mailing of the international search report

02. 05. 96

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## INTERNATIONAL SEARCH REPORT

International Application No

PC1/US 96/00830

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